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Unemployment and the future

Most Western governments are seeking separate solutions for unemployment. They should do more to prove to the jobless that there will be a future, and should agree how to bring it about.

The long recession in the industrialized West has now brought its most daunting and predictable consequence: unemployment. In Britain, the total now out of work exceeds 3 million, or close on 10 per cent of the potential workforce. In West Germany, after several resilient years, the jobless rate is now pushing 7 per cent. In the United States, the growth of unemployment has been even sharper. Even Japan has not escaped unscathed. So it is inevitable that there should be great cries of protest at this state of affairs, and that governments everywhere should be under pressure to modify their monetary policies, devised with varying degrees of good sense to counter rapid inflation. (Reagonomics, devised in Washington with the different objective of unshackling industrial production, has had the same effect because the hard-headed New York money markets have agreed to let the Administration and others borrow money only at deflationary interest rates.) Politically, such pressures cannot indefinitely be resisted. In Britain, it is well remembered that the Heath government embarked in 1973 on its ultimately disastrous policy of reflation because unemployment had exceeded the daunting figure of one million. Although all governments except the French say they will continue to regard unemployment as a kind of lesser evil, the danger is that they will hurriedly change course for the wrong reasons, and at the wrong time.

Unemployment at the rates now customary is naturally hard for even those still at work to stomach. For those without jobs, a large proportion of the younger of whom have never worked, unemployment is a personal tragedy that is only inadequately offset by programmes of social security devised to avoid the impoverishment of the 1930s. So much is commonly accepted, as is the proposition that a high degree of unemployment is a social waste of people's capacity and willingness to work. Yet the present plight of a substantial proportion of the working population in advanced societies is not merely an inevitable but an intended consequence of economic policies now in force, which have in turn been forced on governments by events in the past decade and in particular by the substantial increase of the international price of oil in 1973 and 1979. To pretend otherwise is to suppose that the rules of simple arithmetic can be suspended at will — which is not to say that there is nothing that can be done.

The first need, however, is to be clear where the present trouble has come from. Compared with the happy years before 1973, oil consumers are now paying more than \$200 extra for each tonne of oil they use. For many, this entails a straight transfer of resources to the oil-producing states, which may either decide to convert their funds into a claim on the resources of the oil consumers or, alternatively, may choose to lend back the money through the banking system. Either way, customers for oil on the international markets can have hoped to weather these storms without impoverishment only by increasing their production and therefore their productivity. Japan and West Germany honourably followed that principle for several years. Elsewhere, governments chose to follow their voters' wish that the truth should be concealed from them; inflation gathered pace and had then to be countered by deflationary policies which reduced people's capacity to buy what they think they need and which in turn have now cast their shadow on the economic stalwarts of the West. This argument applies even to Britain, outwardly self-sufficient in oil, because the once cheap oil from the Middle East

has been replaced by expensive oil from the North Sea, won only by capital and resources that might otherwise be used for more productive purposes.

For those who would cure unemployment quickly, the snag is that increased productivity is the enemy of job creation. The dilemma for Western governments is that the continuation of the recession will improve the former — even in Britain, productivity in manufacturing industry increased by more than 7 per cent last year — but will increase the unemployment queues. And the opposite is true — reflation would reduce the immediate misery of unemployment but jeopardize the long-term health of the economy. So are governments irretrievably boxed in? Not necessarily. Those most seriously afflicted are right to resist demands that jobs should be "created" in uneconomic pursuits but, on recent form, cruelly indifferent to the need to explain how the unemployment problem may eventually be solved. People who have just lost their jobs are understandably unwilling to accept that their misfortune must wait while inflation is contained. So what should governments attempt to do?

First, domestically, there is an urgent need that people displaced from jobs that have disappeared should be given a tangible and imaginative opportunity to prepare for the more productive world in which they may yet have the good fortune to live. Although the British government, for example, now plans to spend more generously on training programmes, and is in particular planning to offer every school-leaver who fails to find a job some structured opportunity for training at work, there is nothing to suggest that the results will generally be to make people more employable in the years ahead. By the same tests, all governments must recognize that the key to long-term prosperity — production, productivity and employment all high — is a more effective international division of labour than at present. Here too, unfortunately, the omens are not cheerful. Protectionist tendencies are increasing, with governments seeking to shield from overseas competitors industries that will never make economic sense. Is there a chance that the folly of these policies will be openly acknowledged, as it should be, at the "economic summit" advertised for June?

While they are about it, the governments in June should tackle the still more tricky question of how collectively to manage the transition from where we are to where we hope to be. What criteria will be used for telling when it will again be safe to let up in the battle against inflation? By what common criteria should governments decide that the time has come to invest in public works, or to allow commercial companies to borrow money at more modest interest rates? And by what agreed rules should the value of national currencies be determined and then adjusted? Putting these questions to many of the more orthodox turning up in June will be a little like asking a gathering of churchmen to define the circumstances in which sin is acceptable. In reality, the challenge is not so great. For the agreements negotiated towards the end of the Second World War at Bretton Woods in the United States, which were the basis of international financial relations until they collapsed between 1969 and 1971, were themselves an awkward adjustment between financial probity and an imperfect world. What is needed now is an open recognition that the world is far from perfect, that the governments that inhabit it are even less so and that, nevertheless, they must agree to act in concert.



Goodbye to guidelines

Recombinant DNA guidelines are on the way out. What should be learned from them?

This week's meeting of the Recombinant DNA Advisory Committee at the National Institutes of Health in the United States will be seen as not so much a landmark as a milestone in the brief but eventful history of the most elaborate attempt so far to regulate scientific research. After two years of agonizing in the wake of the Asilomar conference of 1974, government agencies produced rules for research with recombinant DNA which, once promulgated, were almost as quickly dismantled. Already, the guidelines in force are a pale shadow of the tough regulations that caused many professional people to protest that governments were planning to interfere with research — and many members of the lay public to complain that the controls were entirely inadequate. But on present form, the whole apparatus of regulation will have all but disappeared within a decade of being proposed. Has the whole exercise been a waste of busy people's time? What can be learned from it?

That busy people's time has been occupied is of course beyond dispute. People who might have been cloning some gene, or setting up some new biotechnology company, have instead been required to turn up at meetings of advisory and safety committees. There is probably also something in the complaint that important experiments were delayed by the lack of facilities necessary for compliance with the guidelines, or by the diversion of creative people's talents to the development of organisms even less capable of survival in the human gut than the standard laboratory strains of *E. coli* K12. So, now, there are two commonplace appraisals of the experience of the past few years. Some workers in the field claim that they have said from the beginning that the potential dangers of recombinant DNA research were exaggerated, and that the regulations were pointless and a needless interference with free research. Others, more circumspect but also more cynical, say that the guidelines were an unfortunate necessity, an exercise in public relations whose value is attested by the way in which the public clamour about the hazards of genetic manipulation has died down. The truth, however, lies with the smaller company of professionals who acknowledge that the guidelines were both necessary and valuable, that experience of their operation has been instructive and that the history of the recent past should be remembered well.

The argument is simple and straightforward. At Asilomar and in the months that followed, many people in a good position to know the truth held that the hazards for the general population of the construction of novel organisms could be serious. The hazards were always hypothetical, involving processes not then (or since) demonstrated in the real world. Those who claim to have known all along that the guidelines were unnecessary did at the time argue that organisms encumbered artificially with foreign genes were unlikely to be effective competitors in tissues which they happened to infect, but were too scornful of the now common difficulty of deciding how to assess a large danger which has only a very small probability. In the event, the erosion of the guidelines promulgated by the National Institutes of Health (and analogues elsewhere) has been made possible by scientific rather than philosophical developments — the discovery that the replication of genes in eukaryotic cells is more complicated than in bacteria for example. In 1976, the decision that there should be guidelines of some sort was not merely prudent but responsible. So, now, is the general agreement that the guidelines may be further attenuated without risk.

For the most part, the guidelines have also been well kept, even by the sceptics. The few known transgressions, in the United States and elsewhere, appear to have been engendered as much by frustration at the differences in guidelines applying to competing laboratories as to deliberate attempts at cheating. In the United States, the dominant role of the National Institutes of Health in supporting research must have been a powerful deterrent —

which is why, in the history of this period, the failure of successive Administrations to apply to industrial laboratories the sanctions applicable to academic laboratories will seem odd, to say the least. Where guidelines have been backed by legislation, as in the United Kingdom, the courts have not been packed with recalcitrant molecular biologists. The scientific community can claim credit on this score.

So what should happen now? Should the guidelines simply be torn up, and the accompanying bureaucratic apparatus dismantled? That would be to go too far. To the extent that the foreseeable hazards of recombinant DNA research have become no more than coextensive with the risks of working with whatever naturally occurring organisms are involved, the existing rules for research with pathogenic organisms are sufficient. Separate rules for recombinant DNA research are therefore unnecessary, while voluntary compliance with further attenuated regulations will be meaningless. Yet the issues which have been raised in the past several years are interesting and have also made a mark on the public mind. What is needed, now, is a periodic and public reassessment of the risks, hypothetical though they may still be.

For laboratories, the most important procedural discovery since Asilomar is that local safety committees have not turned out to be the interfering busybodies they were feared to be. Even when (as required by the National Institutes of Health) lay members were appointed, the outcome has more often been public enlightenment than outside intrusion into the conduct of research. Indeed, the precedent of local safety committees including members from the nearby community is one that should be more widely adopted in other fields, for example where laboratories have an interest that they should be known to care for their experimental animals — and that the general public should be sensible of their dilemma. It would be no harm if this institution were the chief monument to the guidelines period.

Maths without teachers

British schoolteachers cannot teach mathematics properly, but nobody is surprised.

How should mathematics be taught? And by whom? These questions have perplexed the British educational system ever since it acknowledged, in the 1950s, that everybody profits from education, of which mathematics is an essential ingredient. In despair at not knowing how the questions should be answered, in September 1978, the then British government set up a committee under Dr W. H. Cockcroft, Vice-chancellor of the New University of Ulster in Coleraine, to suggest what might be done. The committee's report now published (*Mathematics Counts*, HMSO, £5.75) is as well polished as the time it has taken to prepare requires in decency that it should be. It is also full of sensible suggestions for the better organization of mathematics teaching in British schools. But on the main issues, the report is muffled.

The trouble with mathematics teaching is that the shortcomings of one part of the educational system are visited upon all others. University courses in mathematics, notoriously arid, are also the chief source of teachers in secondary schools who are saddled with responsibility for teaching mathematics to intending teachers in primary schools. Hitherto (and, indeed, until 1983) it has been possible for young people to embark on careers as teachers without having a formal qualification in mathematics at secondary school, even though they will be required to teach mathematics to young children. The result is that mathematics teaching is everywhere tentative — experts in graph theory teach elementary algebra to thirteen-year-olds, inexperienced people wrestle with seven-year-olds on the conundrum of proportionality. The underlying fault, which Cockcroft does not tackle, is that the British educational system makes a fetish of specialization too early, and pays too little regard to general education at all levels. Paying some good mathematics teachers more, and the other stratagems he advocates, may help in the short run. Changing the way the British educate their young is the only long-term remedy.

Making private interests public

California may ask academics to declare all

Washington

Under new regulations proposed by the state's Fair Political Practices Committee (FPPC), research scientists at the University of California will no longer be given a blanket exemption from conflict-of-interest regulations covering other state employees.

Until now, scientists have argued that academic freedom protects them from being required to disclose their personal stake in outside companies with which they may be involved. These ground-rules are now being shifted, largely as the result of several well-publicized cases in which faculty members have profited substantially from such linkages.

Last month, at a meeting in Sacramento, the state capital, the members of FPPC voted by three votes to two to require that any scientist who accepts a research contract from a private corporation must declare to the university whether he or she has a financial interest in that corporation.

The University of California had previously volunteered to introduce an internal system requiring scientists to report potential conflicts of interest to their heads of department. The regulations being proposed by FPPC would not only formalize this arrangement, giving the state the authority to check that such reporting is being carried out, but would also require the university to set up internal review panels at each campus to which conflict-of-interest problems would be referred.

The commission's proposal has been published for public comment before its formal adoption, expected early next month. It does not go as far as some of the university's critics would have liked; in particular, a legal aid group known as California Rural Legal Assistance, which has been leading a campaign against research into labour-saving agricultural machinery at the university's Davis campus, had petitioned the commission to require research scientists to declare all their interests in outside corporations.

In their present form, the regulations would permit a scientist to keep confidential his or her stakes in a private company interested in the same area of research if the research were funded by a public agency. The regulations would therefore be more liberal than the full disclosure required of other state officials.

However, if the regulations are approved — and are subsequently accepted by the state's Office of Administrative Law — it would mean that faculty members on all nine campuses of the University of

California could no longer use academic freedom as a reason for exemption from the conflict-of-interest rules.

The law which covers the regulations was passed in the early 1970s as part of a nationwide movement to "clean up" the actions of state and federal officials in the wake of the Watergate scandals. Its main purpose is to ensure that state officials are not in a position to gain financially through links with outside contractors and other corporations in which they may have a personal interest.

Soon after the law was passed, its application to the state university was challenged by the university's board of regents on the grounds that it infringed the academic freedom of faculty members. At the time FPPC, which was set up to administer the regulations, accepted this

argument and allowed an exemption.

Since then, pressure on research workers to establish closer links with the private sector has increased as both state and federal funding has decreased. The consequent growth of linkages between faculty scientists and outside corporations has been particularly noteworthy in the development and exploitation of recombinant DNA techniques and genetic engineering. Frequently, however, this has led to tensions between academic and commercial pressures.

One campus of the university is already involved in a law suit over the ownership of a cell line passed to research workers in the private sector and subsequently patented; another has told an agricultural scientist working on nitrogen fixation that he cannot both remain principal investigator

Another French director resigns

Molecular biologists in France are sleeping a little less easily this week, following the spectacular resignation of one of their more important political friends, Professor Philippe Laudat, from his post as director-general of the Institut National de la Santé et de la Recherche Médicale (INSERM).

Laudat waited until after the National Colloquium on Science and Technology (see *Nature* 21 January, p.180) before sending his letter of resignation to the Minister of Health, M. Jack Ralite, and the Minister of Science and Technology, M. Jean-Pierre Chevènement. It was against his own "personal ethics", he wrote, after working energetically for the policies of the previous government, to work now for "noticeably different policies".

The ministries appeared surprised. Laudat's resignation was neither desired nor solicited, said a spokesman for the Minister of Health. "All we did was to ask Laudat to take account of the conclusions of the National Colloquium and the regional assizes [which preceded it]."

INSERM will certainly do that now — for the new director-general will be M. Philippe Lazar, who was chief rapporteur for the colloquium. Lazar is 45, studied at the Ecole Polytechnique and is an epidemiologist interested in the social and economic aspects of health. His star rose with the new government, and his appointment may shift INSERM away from its present strong emphasis on basic biology towards the clinical and softer sciences.

The issues are complex, however. Clinical science in France is generally thought to be weak. Those who work in university hospital clinics are supposed to teach, care for patients and somehow also find time for research. The research suffers. Clinicians are sore that the last

government increased INSERM's effort in molecular biology (with an eye on its application in industry) whilst neglecting sciences closer to medicine. There is thus a battle for resources within INSERM between the clinical scientists and the pure scientists, a phenomenon not unknown elsewhere.

However, the matter was complicated by Laudat's creation of international panels of experts, which doled out money for certain priority projects. These panels were outside the normal semi-democratic control of INSERM's partially-elected review committees, and inevitably they tended to favour the successful (usually basic) laboratories, to the neglect of the ailing clinical research system.

The strong French scientific trade unions have long demanded that the international committees should be advisory to, and vetted by, INSERM's elected committees — a procedure which Laudat clearly felt would destroy their effectiveness. Since the unions now have much greater leverage on the government, the fear now is that this kind of democratic reform will go ahead — with the result that INSERM resources will be spread more evenly and thinly — exactly what Laudat was trying to avoid.

But the government — and Lazar — are unlikely merely to capitulate to union pressure. Chevènement, in particular, is well aware of the ineffectiveness of many university hospital clinics, but he puts it down to inefficient structures, in particular the overbearing power of the "grands patrons", the "gerontocracy" that rules French medical laboratories and — according to some — stifles research. This view, together with the minister's determination to boost biotechnology and its base in molecular biology, may in the end refresh French biology rather than set it back.

Robert Walgate

on a university project sponsored by a large fertilizer corporation and help run a private research company in which the same corporation has invested considerable capital (see *Nature* 8 October 1981, p.417).

A representative of the university said last week that the new regulations proposed by FPPC represented an acknowledgement of some of the recent scientific developments affecting university-industry relations "particularly in genetic engineering, which were not foreseen when we went into this business".

The precise form in which scientists will be required to disclose either their own financial interests, or those of their spouse or children, in companies financing their research has still to be worked out by the university. Also under discussion is the way that the new reporting requirements would be policed. Here the conventional procedure at the university is that, although faculty members are under no legal obligation to make details of their outside interests available to the university, they can be barred from promotion or salary upgrading — both of which must be approved by the state — if they do not do as required.

So far, the university has reacted cautiously to the proposed regulations, recognizing that they have considerable support in the political community. One official on the Berkeley campus claimed that, although many faculty members felt they were being unnecessarily penalized for the excessive actions of a few, the general reaction was a reluctant acceptance.

But the debate may not be over. Some faculty members have apparently indicated informally that they may challenge the new regulations in court on the grounds that they constitute an infringement of constitutional rights. California Rural Legal Assistance in turn is suggesting that it, too, may sue the state if it feels that the regulations are not tight enough.

David Dickson

Polish higher education

Whose pigeon?

Academic freedom cannot be used to combat socialism and to reduce life to anarchy, General Wojciech Jaruzelski told the Sejm (Polish parliament) last week. This was the first meeting of the Sejm since the introduction of martial law. The two-hour speech was intended to justify the drastic measures of 13 December.

On the universities, he noted that the "political tensions" of the past year had involved most of the higher education system, and "weakened the pace" of academic work, creating "painful gaps" in study. The "reinstatement of law and order", however, was now creating conditions for normal work in the universities. "We want to continue the democratization of academic life, to ensure the self-government and autonomy of the

colleges", he said. The new higher education bill should, he said, go ahead.

In spite of his professed support for autonomy, the general suggested that more government control over research would be necessary. In many institutes, he said, the practical results of research are nugatory, and the "discipline of scientific research" must be increased. In this respect he criticized all three sectors of Polish science — the universities, the Academy of Sciences and the "departmental" institutes belonging to the production ministries. Hitherto the "departmental" sector has been given financial priority and better fringe benefits. A main plank of Solidarity's programme for science and culture had been parity between the three sectors. Now, it appears, the three sectors may at least attain parity in the degree of government control. The state, said Jaruzelski, must reserve for itself the supervision of the cost-effectiveness of expensive research, and there should be no repetition in the future of "misguided specialist advice".

This last remark appears to refer to the grandiose investment projects envisaged by the Poland-2000 programme of the Giersek regime — although for the past 18 months the major criticism aimed by the scientists at Giersek was that he commissioned expert reports, and then went ahead with his plans for political reasons in spite of economic and technical indications to the contrary.

The form that the Polish research structure will now take is unclear from the general's speech, although clearly Solidarity's hopes of block-grants for academic research, with autonomy for the universities in the allocation of available resources seem unlikely to materialize. One possibility much discussed during the past 18 months had been the dissolution of the existing Ministry of Science, Higher Education and Technology, and the combination of the higher education sector with the Ministry of Education and Upbringing to form a single education ministry, as existed in Poland before 1972. In that event, a Ministry of Science and Technology would be created with responsibility for all the "departmental" institutes now belonging to the various production ministries, while the Academy of Sciences would continue with its present quasi-ministerial status.

It is not yet certain that these plans will be frustrated. The combination of science, higher education and technology in a single ministry has so far favoured the appointment of a scientist as minister. The new minister, however, appointed last week to replace Dr Jerzy Nawrocki who resigned soon after the declaration of martial law, is Professor Benon Miskiewicz, a military historian, who, until 1981, was rector of the Adam Mickiewicz University of Poznan. The appointment of a minister from the humanities could be the first step towards the eventual reorganization of the ministry. **Vera Rich**

Recombinant DNA guidelines

Only formality

Washington

The Recombinant DNA Advisory Committee (RAC) of the US National Institutes of Health (NIH) is to meet in Bethesda, Maryland, next Monday to decide whether to recommend the *coup de grâce* for federal regulations introduced six years ago to cover research using recombinant DNA techniques.

Many scientists are urging the committee to support a proposal that, as a final step in dismantling regulations initially introduced as protection against potential hazards from such research, would transform them into a voluntary code of practice. Several state and city legislatures, however — most recently the health committee of the California State Assembly — say that if this happens, they may adopt their own stringent regulations.

Judging by past experience, the most likely outcome is that a compromise formula will be worked out by NIH. While reducing the overall stringency of the regulations, this will probably stop short of making them voluntary in the hope of heading off local, more restrictive controls.

Two proposals have been put to RAC as possible major revisions to the NIH guidelines. The more radical suggestion was originally put forward by Dr David Baltimore of Massachusetts Institute of Technology and Dr Allan M. Campbell of Stanford University.

In its current form, which committee members agreed at their last meeting in October should be published for public comment, this revision would reduce the recommended containment level for almost all experiments to P1 physical containment, eliminate all current prohibitions on certain experiments (though retaining two as "admonishments") and abolish the mandatory aspects of the guidelines (see *Nature* 17 December 1981, p.606, for full details).

A less severe revision has been proposed by RAC member Dr Susan Gottesman, of the National Cancer Institute's Laboratory of Molecular Biology. This would also revise and relax the containment requirements, but the guidelines would remain mandatory, and it would still be necessary for universities and research institutions to operate Institutional Biosafety Committees.

Officials at NIH report that, although both proposals have been widely distributed to the scientific community, the response has been much less than that to previous proposals to liberalize the guidelines made when the public debate about the safety of recombinant DNA techniques was at its height.

Some of those on both sides of the debate are clearly coming to an end of their stamina. "I cannot think of any letter that I

A letter to Leonid Brezhnev. . .

Secretary General of the Communist Party
and President of the Council of the Supreme Soviet,
Leonid Brezhnev, Moscow, Kremlin

Sir,

We have followed with great interest your remarks in the press and on television, on the occasion of your latest visit to Bonn, expressing your concern for the maintenance of world peace. This concern is shared by all responsible and thinking people throughout the world, independently of their ideological differences, and we stand with you in your desire to preserve humanity from destruction.

At the time of your visit, however, we were shaken by the news that your country's Nobel Peace Prize winner, Prof. Andrei Sakharov, and his wife, Yelena Bonner, had embarked on a hunger strike of unlimited duration. The ensuing response of the Soviet government, averting the danger to the Sakharovs' lives, was seen by the entire world as an act of humanitarianism. Indeed, we recognize in your government's action an important contribution to détente, a regard for human rights being basic to and inseparable from the search for peace.

The awarding of the Nobel Peace Prize to Andrei Sakharov acknowledges that he engaged himself unequivocally in the interests of world peace, independently of his successes in physics. This recognition of Prof. Sakharov is a tribute also to all our countrymen who, like our own, are filled with the sincere desire for the peaceful coexistence of all nations following the dreadful sacrifices of the Second World War.

In January 1980, Prof. Sakharov was exiled to Gorky, without benefit of a court trial or having been sentenced.

As members of the international scientific community, we feel we cannot ignore the circumstance that Prof. Sakharov, in his two years of exile, has been unable to carry out his scientific work. We see here a clear threat to the freedom of scientific endeavour, and indeed to the freedom of the human spirit.

We therefore appeal to you to accede to Sakharov's repeated request for a full trial, or to allow him to return to his work at the Soviet Academy of Sciences.

Prof. Dr. Gabriele Taugner

An English translation of a letter which was sent on 29 December last year to Soviet leader Leonid Brezhnev and signed by 136 scientists at the University of Heidelberg, West Germany.

have written that has had any effect at all; those of us who think NIH has been dismantling the guidelines too fast still feel as strongly as ever, but we see no evidence that anyone listened to what we were saying", says Dr Susan Wright of the University of Michigan.

Most of the letters received by NIH's Office of Recombinant DNA Activities from individual members of the scientific community, commenting on the two published proposals, support the more radical revision, which would make the guidelines voluntary. This support, the apparent waning of public concern over the potential hazards of recombinant DNA research, legislative efforts to spur the commercial application of genetic engineering, and the anti-regulatory leaning of the Reagan Administration appear to favour a move towards less control.

The single factor which could prevent this, however, is a resurgent militancy in communities in different parts of the country, particularly where new debates about the safety of the research have been triggered by the ambitious plans of the genetic engineering industry.

Both the Boston Biohazards Committee and the Cambridge Biosafety Committee, for example, have written to NIH expressing their opposition to any move that would make the guidelines voluntary.

In California, the state assembly's health committee has recently held two hearings on the growth of biotechnology companies in the state and the current status of federal safety regulations. The chairman of the committee, Mr Art Torres, says he would consider introducing legislation enforcing the guidelines if NIH made them voluntary.

With a large proportion of the nation's genetic engineering research taking place in California, threats of state legislation are

not being treated idly by NIH. In the past, university scientists have made strenuous efforts to prevent Congress from enacting legislation that would turn the NIH guidelines into broad legal requirements; indeed, this has frequently been the motivation behind NIH's agreement to accept tougher restrictions on the research than it would have liked. **David Dickson**

Lead additives in petrol

New offensive

A well-organized campaign to persuade the British government to ban lead in petrol was launched in London last week. The Campaign for Lead-free Air, or CLEAR, claims already to have 130 Members of Parliament, 8 national organizations concerned with environmental and health matters and 21 scientists and clinicians who are convinced by epidemiological studies or their own clinical experience that low blood lead levels harm the mental health of children. The chairman of the campaign, Mr Des Wilson, is a seasoned campaigner whose organization to put forward the case of homeless people, SHELTER, was prominent in the late 1960s.

Lead in petrol has been in and out of British politics since 1980 when a working party under Professor Patrick Lawther found no conclusive evidence that low concentrations of lead in the blood are detrimental to health or that lead in air is a major contributor to increased blood lead concentrations. In the light of the Lawther report, the government's decision last May to reduce the lead content of petrol from 0.4 to 0.15 grammes per litre by 1985 was a surprise.

CLEAR's objectives are to persuade the government to introduce the reduction

earlier than planned and to pass legislation requiring all new cars sold in Britain after 1985 to run on lead-free petrol. The pressure group will also be pressing for a lower excise tax on lead-free petrol and for it to be on general sale by 1985 at the latest.

The campaign, which has received some money from its trustees, hopes to raise £250,000 mainly from public donations. Some of the money will be spent on public education and monitoring the government's programme to reduce lead pollution. The plan is to send out teams to investigate, for example, the effectiveness of the government's programme to increase public awareness of the hazards of lead in old paint and whether car manufacturers are following government instructions to reduce the amount of lead in solder. CLEAR also hopes to support academic research on the effects of low lead levels in the body and to monitor the level of lead in air.

At the launch last week, the organizers described two recent studies implicating low levels of lead in the body as harmful to health. D.A. Otto and colleagues in California, whose study was published in *Electroencephalography and Clinical Neurophysiology* 52, pp.229-239, claim to have found a link between abnormalities in the electroencephalograms of sensory stimulated children aged 1-6 years with blood lead levels as low as 150 microgrammes per litre. Dr Fraser Alexander, a paediatrician from Newcastle and a member of CLEAR's scientific and medical advisory committee, reported his as yet unpublished study implicating low blood lead levels in pregnant women with malformation of the fetus. **Judy Redfearn**

Satellite communications

Up for grabs

Washington

It seemed a good idea at the time; the RCA Corporation, besieged by more than 50 potential customers eager to lease space on its latest telecommunications satellite, Satcom-IV, successfully launched last month, had previously held a public auction for bids to lease seven separate transponders (frequency-shifted radio relays). The response was even better this time. With the auction being held in the Manhattan Galleries of Sotheby Parke Bernet, it attracted a flood of national publicity; the transponders were sold at between \$10.7 and 14.4 million for leases that run until 1988, resulting in a total sale of more than \$90 million dollars.

It was a bit too good to last. On Thursday the Federal Communications Commission (FCC) ruled that the auction was illegal, since it resulted in different prices for leases being paid by different buyers, in violation of federal regulations which require no discrimination. As a result, negotiations with the seven successful bidders have been suspended

until a new way of allocating the leases has been worked out.

However, FCC did not rule out the auction idea completely, and has invited the company to make alternative proposals for selecting bidders that would not contravene federal regulations. One is that sealed bids should be invited, and leases awarded to the seven highest bidders at the price offered by the lowest of the seven.

The dispute between RCA and FCC over how to allocate the leases reflects a gradual shift in attitudes towards the regulation of access to telecommunications satellites. In the early 1970s, when the satellites were first coming into service, the federal government introduced strict regulations to prevent manipulation of the market.

In practice, this goal has been achieved through classifying companies such as RCA which launch the satellites as "common carriers" which are required to offer leases on their transponders on a first-come, first-served basis at prices that are strictly regulated by FCC. The procedure has been effective in protecting smaller users from being squeezed out of the satellite communications market; it has also made it difficult for companies to stockpile transponders, even if they have no immediate need for them, in order to exclude their competitors.

Recently, however, concern has been growing about speculators who have bought leases at the regulated prices and resold them at a considerable profit. In addition, the growth of available space on satellites has led the industry to argue that tight federal regulation is no longer needed. The cause of deregulation of the telecommunications satellite industry has also been embraced by the members of FCC, which in a 7-0 vote last week agreed to publish for public comment new rules which would allow companies operating satellites to negotiate sales directly with private customers.

Satcom IIIR, launched successfully from Kennedy Space Center in November, contains 24 transponders, eight of which were reserved for those who had bought space on the ill-fated Satcom III which failed to go into orbit after its launch in December 1979.

Both Satcom IIIR and Satcom IV are dedicated to use by cable television companies. The satellites are intended to transmit programmes to local television operators, which would then distribute them to individual homes. Since an antenna can only pick up signals from a single satellite at any one time, the companies have been keen to band together rather than lease space on other commercial satellites, such as Westar 1 and Comstar D2.

Among the companies which will have to renegotiate the transponder leases with RCA is RCTV, a joint venture of RCA and the Rockefeller Center Inc., which has announced ambitious plans to distribute

Cashing in now

British Telecom, exercising its right under last year's Telecommunications Act to form partnerships with commercial enterprises, has launched a company to exploit spin-off from research at its laboratories at Martlesham Heath, Suffolk.

The plan is to set up small venture companies to exploit ideas, although patents may be available under licence to existing manufacturers. The new company, Martlesham Enterprises, has been set up with £250,000 of issued ordinary share capital, the shareholders being British Telecom (30 per cent), Electra Investment Trust (25 per cent), Lazard Brothers (20 per cent), Raeburn Investment Trust (managed by Lazards) (20 per cent) and Thompson Clive and Partners (5 per cent).

Research at the Martlesham Heath laboratories covers optical fibres, semiconductors, video-phones, slow-scan TV, speech synthesis and public viewdata systems. Martlesham Enterprises has already identified five ideas which could form the basis for new manufacturing companies, but so far only one of them, a new materials process for semiconductors, has been developed to any significant extent.

Martlesham Enterprises plans to hold a financial interest in new operating companies, giving its shareholders first option to put up new capital. Attractive terms are to be offered to inventors who leave the laboratories to help set up new companies. Interest on profits will, as normal, return to the shareholders. It is hoped that the operating companies will grow to the point where they can be launched publicly, when Martlesham Enterprises will decide whether to retain a financial interest or dispose of them.

Judy Redfearn

entertainment programmes through cable television. The company has already agreed to buy the first rights of British Broadcasting Corporation programmes for transmission in the United States.

Meanwhile, in a separate move illustrating the social and political possibilities being opened up by satellite telecommunications technology, four labour unions have joined with the American Federation of Labor/Council of Industrial Organizations to arrange for the broadcast over public television of two films on safety and health whose distribution had been suspended by the Department of Labor's Occupational Safety and Health Administration. The unions have made use of a 1978 amendment to the 1934 Communications Act which allows groups outside public broadcasting to rent time on one of the four transponders which the Public Broadcasting Station (PBS) leases on the satellite.

David Dickson

Technical change in France

Central theme

The French government is to set up a centre for social and systems research on science and technology, inspired to some extent by the Brookings Institution of Washington and, in Britain, the Technical Change Centre in London and the Science Policy Research Unit (SPRU) in Sussex. But in the Paris institution there will be a difference: it will be strictly linked to, and under the direction of, the Ministry for Research and Technology, and will thus lack the political independence of its models.

The Center for Advanced Technological Systems Study (CATSS), to use its Americanized name, (CESTA, Centre d'études des systèmes et des technologies avancées, in French) should be in full swing by 1984 with a budget of around 40 million francs and a staff of 40 "scientists and engineers".

The creation of CATSS is the immediate result of a report to Prime Minister Pierre Mauroy by Dr Joël de Rosnay, director for research applications at the Institut Pasteur (where Francois Gros, science adviser to the Prime Minister, was until recently director). De Rosnay recommended that a centre like CATSS be established "to study the industrial, social and cultural change induced by advanced technological systems (such as microelectronics, biotechnology, robotics and solar energy)".

The questions are political and involved, the report notes, and it claims that in France "there are traditional difficulties" in thinking in terms of complex systems and networks. CATSS must overcome these difficulties.

As a government agency in a government devoted to industrial development through technology, CATSS may find its emphasis lying more frequently on innovation strategy in key industries than on social impact *per se*. The emphasis of the report is on missed, or botched, industrial opportunities: Concorde, an "indisputable technical success" marred by a failure to anticipate economics, competition and environmental impact; microelectronics, insufficient in France because opportunities to develop liquid crystals and integrated circuits were not taken up; and biotechnology, limited by the lack of researchers and engineers trained in the life sciences.

Despite its government links, the centre will be open to scientists, industrialists, union leaders, parliamentarians and local politicians. It will also collaborate with outside institutions (including, perhaps, Brookings, SPRU and the Technical Change Centre).

CATSS will have a strong training function as well as undertaking research, experimenting with new educational technology and communications systems,

and helping to "diffuse" new technology. Thus the concept appears to be of an intellectually and politically sophisticated institution to assist the country's technological development: one which will be able to help industry to make its forecasts and plans, but will, at the same time, take into account likely social impacts and obstacles. CATSS's first programmes begin next month — training programmes in biotechnology and micro-electronics. A director for the centre is expected to be appointed before the summer.

Robert Walgate

Fluoridation in Scotland

Days in court

A legal trial that will decide whether Strathclyde Regional Council becomes the first Scottish water authority to add fluoride to its water supply has now passed its 130th day, making the record books as the longest ever court case to be heard in Scotland. The Edinburgh Court of Session, Scotland's High Court, has become a battleground for fluoridation and anti-fluoridation interests.

The case, *McCull v. Strathclyde Regional Council*, opened on 23 September 1980, and was originally expected to last twelve weeks. The eventual cost is estimated at £1 million, which would make it the most expensive civil case in Scottish legal history. The costs of Strathclyde Regional Council will be met by its four area health boards. The expense of an expert witness can be as much as £450 a day and many have come from as far away as the United States and Australia. If the case lasts for 150 days, as it may well do, lawyers' costs alone could exceed £60,000.

The issue at stake is twofold — the legal right of the council to add a trace of fluoride to the water supply and the medical effects of fluoride. In 1977, the council voted by a majority of 43 to 42 to build a £2 million plant to introduce fluoride into the water supply. Mrs Catherine McCull is contesting the decision, claiming the right of herself and her grandchildren to drink unfluoridated water on the grounds that fluoride is not only useless but can cause cancer and other diseases. The hearing is before Lord Jauncey.

The evidence has been intricate and technical. Mrs McCull appeared in the box only on the first day. The medical issues have focused on the contention that fluoride can cause cancer. Sir Richard Doll for example, has so far given evidence for two weeks. It is expected that Lord Jauncey will take months to reach a judgement.

Evidence against fluoridation has been given by Dr John Yiamouyiannis, formerly of the National Health Federation organization and a member of the US National Health Action Committee, and Dr Dean Burk, a retired National Cancer Institute laboratory scientist. In some ways the

Scottish trial is a re-run of the hearing in West View, Pennsylvania in November 1978, when Drs Yiamouyiannis and Burk also argued that there is a link between fluoride and increased cancer mortality rate. Dr Burk has separately told the US House of Representatives that fluoridation is "socially imposed murder".

In Pennsylvania, Judge Flaherty, then a lower court judge, issued an injunction ordering West View Water Authority to cease water fluoridation. His decision, however, was appealed against on grounds of jurisdiction. In January 1979, after a review of the evidence by the Department of Environmental Resources, the water authority was ordered to continue to fluoridate. The Minnesota Governor's Commission, appointed in 1978 to study the health effects of water fluoridation, examined the same body of evidence as Judge Flaherty and concluded that "an association between fluoridation and cancer has not yet been shown".

Fluoride has been added to water supplies in Britain since 1957, and 10 per cent of the population drinks artificially fluoridated water (in the United States 44 per cent of the population drinks treated water). The 1972 Water Act enjoins the water authorities to provide "wholesome water", but although 87 out of 90 area health authorities are in favour of fluoridation, in the past 7 years few schemes have been implemented. The water authorities seem reluctant to proceed in the absence of parliamentary legislation.

The decision in Scotland could thus be influential, particularly as a similar case is being prepared in Calderdale, Yorkshire. If Lord Jauncey decides in favour of Strathclyde, the issue of further legal aid for an appeal will arise. If the case goes to the House of Lords, it may take several years for a final decision. And neither side may be able to claim a victory — if, for example, the judgement is that fluoride is beneficial to dental health but that water authorities do not have full jurisdiction to determine what goes into the water supply.

In Strathclyde, the irony is that local elections early this year could render any judgement nugatory: a new council could vote to reverse the fluoridation policy.

Jane Wynn

Rubik's cube

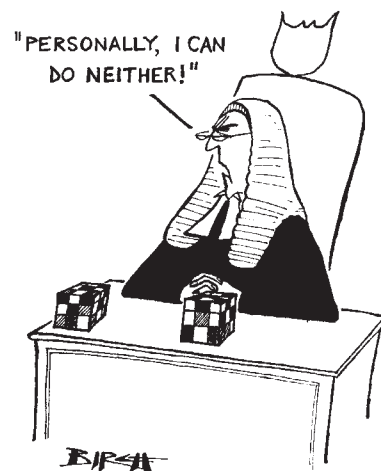
Puzzles galore

A decision is expected this week from the British high court hearing of a copyright and passing-off suit brought by the Politehnika Industrial Corporation of Budapest, manufacturers of the original Rubik's cube, against Dallas Print Transfers of London, importers of an alleged copy known as the "Wonderful Puzzler", produced in Taiwan.

The case, initiated by an injunction from Politehnika last March, has turned on two main issues. As far as copyright is con-

cerned, the emphasis has been on the internal structure of the cube, involving comparison of production blueprints, while the passing-off charge is based on claims that the puzzler has on occasion been marketed as the Rubik cube.

The dual nature of the case corresponds appropriately to Dr Erno Rubik's own dual interest in the cube — structure and concept. A lecturer in architecture at the Budapest Academy of Applied Arts, he was, he says, originally interested primarily



in the internal mechanism of the cube which allows each face, consisting of a 3×3 array of cubelets, freely to rotate either clockwise or anticlockwise. Only later did he decide to communicate his pleasure in the new device to others by offering it for commercial construction.

Rubik says that other international contributions to the booming market in logic toys — pyramids, spheres and the like — embody essentially the same principle as the cube, since the various sections are displaced without changing the over-all shape of the assemblage. The one major exception, known as the "tower", which exists in two contending Hungarian versions, is, in his opinion, simply the old "15 puzzle" invented in the 1880s, rolled up into cylindrical form, since it incorporates a vacant parking spot into which an element can be moved during the transfer process.

The popularity of the cube, which Rubik himself cannot adequately explain, has made him reputedly the seventh richest man in Hungary and won for him a "Gold Medal of Labour" for his services to Hungarian industry, and has had a number of unexpected spin-offs. The possible and impossible configurations of the cube, Rubik said last December, were attracting attention from physicists as a means of modelling the properties of quarks.

In Hungary, the unprecedented boom in the toy industry generated by the cube has highlighted a number of defects in the New Economic Mechanism, and seems to have been in part responsible for the recent relaxation of the rules governing small enterprises.

Vera Rich

CORRESPONDENCE

Soviet sanctions

SIR — I welcome your leading article of 7 January (p. 1) and in particular your criticism of the suspension of the exchange agreement between the United States and the Soviet Union.

Such a suspension must surely weaken world peace. The merits and demerits of unilateral nuclear disarmament are much debated but the prior need is for a better understanding between peoples of different cultures — in this case the United States/United Kingdom and the Soviet Union. The risk of scientists "having their brains picked" surely matters less than the potential benefits from better knowledge of human beings of the "other" culture — their hopes, fears and hang-ups.

As a first line of defence for international understanding and peace, scientific and other exchanges can be good investments and should be fostered, not reduced. Their costs, if prohibitive to self-financing individuals, are infinitesimal compared with those of armaments.

H.J. KILLICK

Abingdon, UK

Ethnic journal

SIR — H.H. Hirschhorn (*Nature* 14 January, p. 92) appears to have misunderstood the "small niche" to which *Journal of Ethnopharmacology* is trying to lay claim, even though my review quoted the aims of the journal in full. Rather than being another journal of pharmacognosy, which I would accept could appear to be of greater importance than all the drugs for the cardiovascular system, *J. Ethnopharmacology* specializes in the use of drugs by ethnic cultures. How does the importance of "ceramic use for prehistoric Datura use in N. America" compare with a typical paper from *Journal of Cardiovascular Pharmacology*?

The number of reprint requests might reflect more accurately the obscurity of the journal and difficulty in finding it in a library, than the importance of the subject.

HILARY G. PICKLES

St Bartholomew's Hospital,
London, UK

Anthrax island

SIR — The paper by Manchec *et al.*¹ provides the first published quantitative information about the contamination of Gruinard Island with anthrax spores. In view of the public interest and concern that have been expressed, this is welcome. Nevertheless, the information given is meagre and the main tentative conclusion questionable.

Since no accurate viable counts of spores were made on samples taken before those reported on in the paper, it is impossible to estimate the time course of the contamination. The authors consider that its present extent warrants their taking seriously van Ness's assumption² that significant multiplication of anthrax bacilli may occur in the soil. However, neither in that paper, nor in a series of earlier papers, does van Ness cite experimental evidence to justify this assumption. In support, he quotes a paper by Minnett and

Dhanda³. But those authors actually come to the *opposite* conclusion — that the anthrax bacillus does *not* behave as a saprophyte in nature.

In the past, I have exhumed and examined cadavers of cattle that have died of anthrax, but I failed to isolate anthrax from any, even from one buried as little as three months previously. The conditions — high ambient temperatures and the availability of blood and tissues from the cadavers for the nutrition of the anthrax bacilli — resembled those postulated by Manchec *et al.*¹ as being likely to promote an increase of bacilli and spores in the soil. However, those familiar with the epizootiology of anthrax tend rather to regard such conditions as favourable to the increase of soil microflora inimical to anthrax organisms; a view which practical experience reinforces.

There is little experimental evidence to support either van Ness's view or the more conventional opinion that anthrax does not behave as a saprophyte. Unfortunately, incentives to investigate the ecology of the anthrax bacillus have diminished — despite its intrinsic interest — because of the ease with which anthrax can be controlled by the vaccine I developed⁴.

Attention should be paid to studying factors which could be responsible for the exceptionally long survival of the contamination of Gruinard Island. Is this related, for example, to the form in which the spores were distributed; to their number; to the character of the soil or of the soil microflora; to the lack of husbandry; or to climatic conditions? It would be deplorable if the special circumstances on Gruinard Island were not used to provide answers to these neglected ecological questions, and particularly to the question as to whether the anthrax bacillus can multiply sufficiently in the soil for this to be a significant factor in the epizootiology of anthrax. Practical experience in the control of anthrax does not support this view.

M. STERNE

Sarisbury Green,
Hants, UK

1. Manchec, R.J., Broster, M.G., Melling, J., Henstridge, R.M. & Stagg, A.J. *Nature* 294, 254-255 (1981).
2. Van Ness, G.B. *Science* 72, 1303-1307 (1971).
3. Minnett, F.C. & Dhanda, M.R. *Indian J. vet. Sci.* 11, 308-328 (1941).
4. Sterne, M. *Onderstepoort J. vet. Sci. Anim. Ind.* 21, 41-43 (1946).

No change in RE

SIR — D.B. Jack and M.R. Gregg (*Nature* 22 October 1981, p. 602) have used Flesch's index of reading ease (RE) to suggest that letters to *Nature* became harder to read at some time between 1940 and 1950. Using the first hundred words in each letter as the criterion, the papers analysed from the years 1950 to 1980 were, on average, 15.44 index points more difficult (the harder to read the lower the index).

This conclusion obscures a difference of style between earlier and more recent letters to *Nature*, that of the way in which the opening paragraph is used by authors. Current practice demands that the opening paragraph shall serve two functions, both as introduction and

as a summary of the main conclusions. In its latter function it must often be laden with a burden of latinate technical terms and exactitudes, for those readers who choose to go further. The earlier style demanded simply that the opening paragraph served to whet the appetite and to lead the reader gently to the nub of the matter, eschewing difficulties at first.

I have therefore computed Flesch's RE for the first and second paragraphs of two samples of 20 letters each, culled from *Nature* of October 1981 and of October 1931. If the second paragraph contained less than a hundred words the third paragraph was lumped with it.

	Reading ease		Total text
	Para 1	Para 2	
1931	29.8 ± 4.3	23.7 ± 4.1	26.3
1981	15.1 ± 2.1	21.0 ± 2.4	18.5

This analysis confirms Jack and Gregg's conclusion that the opening sentences today are more difficult than they were 50 years ago. In my sample the difference is 14.7 RE points. The 50 year difference for the second paragraph, however, is a mere 2.7 points which is not statistically significant. In 1931 the second paragraph was on average 6.1 points more difficult than the introduction; today it is 5.9 points easier.

Authors today seem not to be cloaking their findings in obscure language significantly more than they did 50 years ago, except in the introduction.

D.B. CARLISLE

Environment Canada,
Ottawa, Canada

More repeats

SIR — There appear to be two homologous sequences in a single exon of the collagen $\alpha 2(1)$ sequences reported by Wozney *et al.* in *Nature* of 12 November 1981¹.

The sequences occur in a single polypeptide and are apparent in the nucleotide sequence but not the amino acid sequence. A span of 36 nucleotides is repeated with six differences (marked with ×):

```

1242                                1278
GlyAlaAlaGlyProGlyProGlyProSer
GGTGTCTGCTGGCCCTTGGCCCTCTGGTCCAAGT
      XX              X              X
GGCTCTGCTGGTCCCTTGGCCCTGCTGGTCTAAGA
GlySerAlaGlyProGlyProAlaGlyLeuArg
1315                                1351

```

The differences are probably due to post duplication mutations.

The sequences lie 4 collagen units, (Gly-X₁-Y)₄, from each other and contain 4 collagen units each, which makes a 54 base pair (bp) repeat hard to credit. I cannot think of a plausible scheme to yield 36 bp repeats from 54 bp units. Apparently 36 bp were extracted by double unequal crossover and re-appended 36 bp further down the molecule leading to the internal homology.

MICHAEL P. FINERTY

Tucson, Arizona, USA

1. Wozney, J., Hanahan, D., Tate, V., Boedtker, H. & Doty, P. *Nature* 294, 129-135 (1981).

NEWS AND VIEWS

Mantle heterogeneity and isotopes in oceanic basalts

from W.M. White and A.W. Hofmann

THE chemistry of volcanic rocks is strongly influenced by processes which occur during magma genesis and evolution and it is generally difficult to relate it to the chemistry of the rocks' sources. One exception is the isotopic composition of radiogenic elements such as Sr, Pb and Nd, which are thought to be unaffected by most magmatic processes and provide a means of 'fingerprinting' volumes of source materials in the Earth's mantle. It has been known for nearly two decades that the mantle is isotopically and chemically heterogeneous but the interpretation of the geochemical data is not easy because the kinematics of mantle convection remains elusive. Now isotope geochemists and mantle convectionists are beginning to interact and collaborate in order to overcome this difficulty. Two issues remain unresolved — the size of the domains of mantle heterogeneity and their spatial arrangement — is the mantle layered or irregularly heterogeneous?

On the geochemical side, it is well established that volcanic rocks erupted on oceanic islands are apparently derived from a type of mantle fundamentally different from that which supplies magma to most of the mid-ocean ridge system. Second, all of the mantle now involved in mid-ocean ridge volcanism has previously been depleted in incompatible elements (that is, elements having a strong affinity for a melt phase as opposed to the solid). Further, heterogeneity in the mantle is long-lived. Pb isotope data suggest that the various reservoirs have remained separate for a time of the order of 2×10^9 yr, demonstrating that mantle convection does not stir the mantle sufficiently to eliminate the heterogeneity. Thus, and because of the hope that the isotopic data will somehow give clues about mantle kinematics and dynamics, geochemists continue to gather isotopic data on mantle-derived volcanic rocks.

Systematic studies of basalts from the Mid-Atlantic Ridge in the 1970s revealed that, as islands such as Iceland and the Azores are approached, isotopic compositions of Sr and Pb change gradually from those typical of mid-ocean ridge basalts to compositions characteristic

of island basalts, providing evidence of geographically correlated variation in mantle chemistry on a scale of the order of 100–1,000 km. Two recent papers in *Nature* deal with mantle heterogeneity on a local scale. Dupré and co-workers (*Nature* **294**, 552; 1981) report Pb and Sr isotopic data for two well studied areas of the mid-ocean ridge system: the FAMOUS area in the North Atlantic and the CYAMEX area of the East Pacific Rise. Basalts erupted in these two areas have nearly uniform Sr isotopic compositions and though Pb isotope variations exist, they are much smaller than variations observed on larger scales. Earlier studies of Sr and Nd isotopes in basalts from the FAMOUS region also revealed virtual isotopic uniformity.

Machado and his colleagues (*Nature* **295**, 226; 1982) report Sr and Nd data on basalts from the vicinity of the Kane Fracture in the North Atlantic, some 200 km from the ridge. Measurable variations occur in both Sr and Nd, with basalts from the western ridge segment having systematically higher $^{87}\text{Sr}/^{86}\text{Sr}$ and lower $^{143}\text{Nd}/^{144}\text{Nd}$ ratios than those from the eastern ridge segment. The total range of Sr isotopic compositions ($^{87}\text{Sr}/^{86}\text{Sr} = 0.7022\text{--}0.7025$) is small compared with what is normally found in mid-ocean ridge basalts, but the range of Nd isotope ratio is relatively large ($^{143}\text{Nd}/^{144}\text{Nd} = 0.51337\text{--}0.51313$). The Fracture Zone apparently forms a boundary between two geochemical domains in the mantle.

Yet another recent study bearing on the question of the scale of mantle heterogeneity has been reported by Zindler and co-workers (*EOS* **62**, 424; 1981). They find that seamounts in the vicinity of the East Pacific Rise have $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios varying between 0.7027 and 0.7032 and 0.5132 and 0.5130 respectively, and basalts from a single small seamount span this entire range.

Local isotopic variations have been reported from oceanic islands in earlier studies. For example, on the island of Sao

Miguel (Azores), 60 km in its longest dimension, reported $^{87}\text{Sr}/^{86}\text{Sr}$ ratios range from 0.703 to 0.705, while $^{143}\text{Nd}/^{144}\text{Nd}$ ranges from 0.51300 to 0.51276. On Iceland's Rekjanes Peninsula, $^{143}\text{Nd}/^{144}\text{Nd}$ ratios vary from 0.51317 to 0.51299 on a scale of a few tens of kilometres and less, although $^{87}\text{Sr}/^{86}\text{Sr}$ is nearly uniform. Although it is often assumed that Sr and Nd isotopic variations are closely related, these studies, and that of Machado and his colleagues, demonstrate that these ratios, and by inference variations in Rb/Sr and Sm/Nd, may occasionally become decoupled.

The conclusions that may be drawn from the data accumulated so far are: (1) in most regions, especially along the mid-ocean ridge but also on many oceanic islands, isotopic compositions can be mapped, and it is found that the regional variations are larger than the local ones. (2) Nevertheless a few regions, such as 45°N on the Mid-Atlantic Ridge, the island of Sao Miguel and the small seamounts off the East Pacific Rise, do show significant local variability and cannot be characterized by a single isotopic ratio. (3) The local variations generally have greater amplitudes on oceanic islands than on the mid-ocean ridge. (4) The regional variations are also larger for oceanic islands than for ocean floor basalts.

The question of scale is intimately related to the question of origin. A view widely held by geochemists and geophysicists, but by no means universally accepted, is that oceanic islands result from mantle plumes arising from the deeper mantle, while the upper mantle, depleted in incompatible elements through repeated episodes of melt extraction, is the source of mid-ocean ridge basalts. The lower mantle source of mantle plumes may be either primitive, undepleted mantle or subducted oceanic crust. An alternative view is that mantle heterogeneity is created by fluids rich in incompatible elements percolating upwards and metasomatizing the upper mantle; this is supported by evidence from mantle xenoliths in alkali basalts and kimberlites. In this model there is a large potential for creating small-scale heterogeneity if fluid pathways are not uniformly distributed. The plume

W.M. White is in the Geochemistry Department, Max-Planck-Institut für Chemie, D 6500 Mainz, FRG, of which A.W. Hofmann is Director.

hypothesis predicts that oceanic islands are created at quasi-stationary locations and that large quantities of material are advected to the surface in excess of the volume needed to create normal oceanic crust. The existence of isotopic anomalies of large wavelengths and large amplitudes is certainly consistent with this hypothesis, and we have long favoured this idea. However, the hypothesis that isotopic variations are produced by more local phenomena possibly involving metasomatism does not really conflict with this view; rather, it may be the appropriate explanation for many or all of the small-scale isotopic variations observed. Indeed, it seems unlikely that the mantle plumes can explain everything. Surely not all seamounts are former or present sites of mantle plumes, nor is it clear, for example, how the isotopic variations near the Kane Fracture Zone could be related to a mantle plume.

We believe, perhaps unimaginatively, that in order to improve our understanding of mantle heterogeneity we do need to accumulate a lot more isotopic data on more oceanic basalts rather than wait until evidence of a different kind or insight provides a new impetus. Further study of the mid-ocean ridge system will be essential if the questions about the chemical structure of the mantle are to be resolved. Is the well studied North Atlantic Ocean anomalous because of the presence of the very large Iceland and Azores anomalies? Do other areas similar to 45°N exist or is the mid-ocean ridge system more nearly uniform away from oceanic islands? What is the total volume of isotopically anomalous basalts? Do all postulated plumes have large-scale isotopic gradients associated with them? Can the apparently randomly distributed small-scale variations also be mapped with improved coverage? Do major fracture zones, such as the Kane, form boundaries between geochemical domains in the mantle? To reconstruct the distribution of source materials and their movement in the mantle, we still need much more complete isotopic maps of the ocean floor and the oceanic islands than those so far compiled.

What is required is a programme of intensive mid-ocean ridge 'geochemical mapping' similar to that already carried out in the North Atlantic. We believe this would most profitably be accomplished through dredging rather than drilling because of the higher sample quality and the substantially lower costs. Drilling of seamounts and similar anomalous features, already begun by the International Phase of Ocean Drilling (IPOD), should continue. We think of this 'fingerprinting' of source materials in the Earth's mantle using isotopes as perhaps being similar to fingerprinting the oceanic crust using magnetic anomalies, the meaning of which remained obscure until there were enough data for them to be interpreted by simple visual inspection. □

New measurements near the Gulf Stream

from W.J. Gould

THE Gulf Stream is, to the layman, a well known ocean circulation feature responsible for the temperate climate of Western Europe. It would therefore seem surprising that oceanographers should be in any doubt about the structure and dynamics of the Gulf Stream in view of studies that have been made over many years¹⁻³. However, until recently, progress in the field has been restricted by the difficulty of measuring directly the current in the strongest parts of the Gulf Stream. Now this problem has been overcome and a recent paper⁴ sheds light on the relationship of the Stream to its recirculation and on the Gulf Stream's inherent variability.

The Gulf Stream is the intense (surface currents of 3 knots (150 cm s⁻¹) or more) narrow current that flows northwards along the eastern seaboard of the North American continent from the Florida Straits to south of the Newfoundland Grand Banks. North of Cape Hatteras the Stream begins to meander, with the envelope of the stream tracks spreading like a fan as it turns eastwards away from the continental slope and past the New England seamounts. The intense current is associated with the boundary of the warm Sargasso Sea water, which fills much of the western North Atlantic, and the colder 'slope water' adjacent to the continental slope. (The current axis is often defined as the locus of positions at which the temperature at 200 m depth is 15°C.)

East of the Grand Banks the current and its associated temperature structure rapidly lose their identity and the course of the Gulf Stream becomes diffuse. It is at this point that the current is considered to form two main branches, the North Atlantic current which heads northeastwards towards Europe, and the Gulf Stream recirculation which turns south and eventually westwards to rejoin the Gulf Stream and complete the subtropical Gulf Stream gyre.

Until comparatively recently the view of the Gulf Stream system (Gulf Stream, North Atlantic current and recirculation) has been derived solely from observations of the density field (from temperature and salinity observations) which are then used to derive the geostrophic flow field (based by definition on the assumption of a balance between the horizontal pressure gradients and the Coriolis force). A typical view⁵ shows a Gulf Stream system confined almost entirely west of the Mid-Atlantic Ridge and characterized by two anticyclonic (clockwise) gyres penetrating throughout the entire water column. The main gyre is that of the Gulf Stream and its recirculation and the secondary one lies to

the east of the Newfoundland Grand Banks. But the interpretation of the temperature and salinity data in terms of two gyres is controversial⁶. The calculation of geostrophic currents provides only a current profile relative to some arbitrary reference level, usually at depth, and thus while the near-surface circulation may be adequately described the deep current field may not.

A possible explanation of the exact nature of the circulation may come from direct observations of currents either by moored current meters (eulerian measurements) or by the tracking of drifters (lagrangian measurements) at the surface or at depth. The strength of the Gulf Stream has so far precluded the use of moored current meters in the upper part of the water column near the Gulf Stream axis although they have been used extensively north and south of the Stream and in the deep ocean below it. Lagrangian measurements do not have the same restriction and recent technology presents new possibilities for investigating the Gulf Stream system.

Neutrally buoyant floats are instrument packages designed to be less compressible than seawater. Thus, as they sink, they gain buoyancy relative to the surrounding water until by suitable ballasting they may be made neutrally buoyant at some pressure level. For many years since their development in the 1950s these floats were tracked acoustically from attendant ships^{7,8} but their use was restricted to the duration of a typical research cruise (a few tens of days). In the late sixties and early seventies a series of neutrally buoyant floats with lifetimes of two or three years were tracked in the southern Sargasso Sea^{9,10} using low-frequency sound signals (400 Hz) received at shore-based listening stations in the Caribbean. The necessary acoustic propagation over distances of the order of 1,000 km is achieved by means of a minimum near 1,000 m depth in the vertical profile of sound speed in the ocean which acts as a wave guide. This is known as the SOFAR (SOund Fixing And Ranging) channel and the long-range floats as SOFAR floats.

The technique was initially restricted to areas within range of the fixed receivers but the development of moored autonomous listening stations (ALS) in which signals from the SOFAR floats are received, processed and stored has raised the possibility of deploying SOFAR floats almost anywhere in the ocean.

A recent paper by Schmitz *et al.*⁴ provides some of the first direct measurements of currents in the strongest parts of the Gulf Stream and helps explain the nature of its circulation. The float tracks described are from depths of

W.J. Gould is at the Institute of Oceanographic Sciences, Brook Road, Wormley, Godalming, Surrey GU8 5UB.

700–2,000 m and have been considered to provide eulerian estimates of current from the areas through which they passed. These have been compared with eulerian measurements of currents from either side of the Gulf Stream. (There are some fundamental difficulties in the interpretation of the float tracks. Since they depend for their neutral buoyancy on the ambient pressure they tend to follow isobaric surfaces and so do not stay with a particular body of water, that is, they are not truly lagrangian.)

The float tracks reported exhibit similar complexity in space and time to synoptic views of the Gulf Stream from temperature field maps and from previous current meter records. The kinetic energy of the floats is consistent with an extrapolation of known meridional variations of kinetic energy¹¹ into the region of the Gulf Stream axis. The deeper float tracks do not, however, support the view of a vertically coherent Gulf Stream, floats close to the Gulf Stream axis exhibiting both westward and eastward motions. The tracks of floats in and above the thermocline (depth of greatest current shear) support previously held views of the circulation.

New measurements in the vicinity of the Gulf Stream at both thermocline and abyssal depths raise the possibility of connecting the known (from eulerian measurements) time scales of variability south of the Gulf Stream in the Sargasso Sea recirculation and north of it on the continental slope¹². Subject to the only quasi-lagrangian nature of the float tracks there may also be evidence of the geographical distribution of the recirculation of the Gulf Stream system and a possible resolution of the two gyre controversy.

There are now plans for a wider use of SOFAR float technology. In particular, the next two years should see measurements taken by US, UK and French laboratories near and to the east of the Mid-Atlantic Ridge in an effort to study the paths taken by the North Atlantic current on its eastward path and long-term dispersion in the deep ocean. These projects will use SOFAR floats in areas of more complex sound velocity structure than are found in the western North Atlantic and at much greater depths than previously explored. □

Substorms and the growth phase problem

from S.W.H. Cowley

SPACECRAFT observations over the past decade have clearly demonstrated that large-scale dynamic changes in the magnetic field and plasma populations take place periodically inside the Earth's magnetosphere. These disturbances are termed magnetospheric substorms. They last for 1–2 hours and it is not unusual for several to occur in one day. At the Earth, substorms are associated with brilliant auroral displays resulting from bombardment of the upper atmosphere by kilo-electron volt magnetospheric electrons, and with intense ionospheric electric currents flowing at high latitudes.

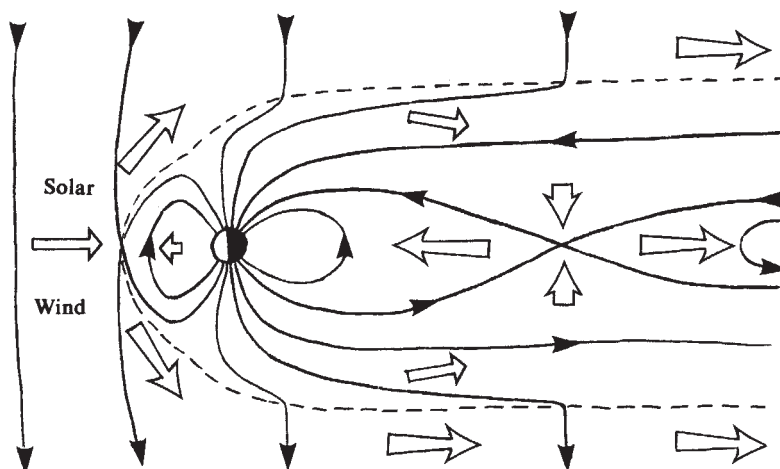
It is generally agreed that substorms tend to occur when the coupling between the magnetosphere and the surrounding solar wind is strong. Magnetospheric coupling to the solar wind causes a large-scale circulation of plasma and embedded magnetic field lines within it (see the figure). When the solar wind magnetic field points south, as shown, magnetic 'reconnection' can occur across the dayside boundary so that the solar wind magnetic field becomes directly connected into the Earth's polar magnetic field. These terrestrial 'open' field lines are then swept downstream by the solar wind flow and are stretched out into a long magnetic tail typically extending to $\sim 1,000$ Earth radii (R_E) on the nightside of the Earth. A similar 'reconnection' process then occurs (at least eventually) in the tail, returning 'closed' field lines to the Earth and thence to the dayside boundary where the process can repeat, as originally described by Dungey in 1961. The important point is that the strength of the cyclic flow depends primarily on the direction of the solar wind magnetic field. Magnetospheric substorms occur preferentially

when the field points south and dayside reconnection and the flows reach a maximum. As the solar wind field turns northwards, both the flows and the occurrence of substorms decrease.

There is broad agreement that once a substorm begins it can be described in terms of auroral brightening and 'break-up', the sharp intensification of ionospheric currents and the disturbances which occur in the magnetic field and plasma in the nightside magnetosphere. However, there are two important and opposing views on the events which lead up to substorms: the 'growth phase' model of McPherron¹ and the 'directly driven' model discussed most recently by Akasofu².

The 'growth phase' picture assumes that the increased magnetic flux transfer from the dayside to the tail which follows a southward turning of the solar wind magnetic field is not immediately matched by increased flux return from the tail to the dayside. As a result, magnetic energy is stored in the increasing field in the tail for perhaps 1 hour or more before substorm onset (the 'growth phase'). At a certain point, however, an instability occurs (substorm onset), resulting in the formation of a new reconnection region about $15 R_E$ down-tail from the Earth. The rapid tail reconnection which then occurs dissipates the stored magnetic energy, feeding it over periods of about 1 hour into plasma energy (the substorm expansion phase). The substorm subsides when the reservoir of available magnetic energy in the tail is depleted, but a new substorm cycle can then start if the solar wind field remains southerly directed.

The 'directly driven' substorm model is based primarily on the close correlation



Cross-section of the Earth's magnetosphere in the noon-midnight meridian plane. The solid lines are magnetic field lines, and the dashed line is the magnetosphere-solar wind boundary which on the dayside of the Earth lies typically at a geocentric distance of $10 R_E$. Solid arrows indicate the flow of plasma and embedded magnetic field lines, and show the convection system set up inside the magnetosphere by the magnetic 'reconnection' which occurs between the Earth's field and the solar wind magnetic field across the dayside magnetopause when the latter is southerly directed.

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observed between substorm and solar wind conditions and postulates that the state of the magnetosphere is determined solely by the solar wind, with a magnetospheric response delay of perhaps a few tens of minutes. The correlation is described in terms of a function of solar wind variables termed the ' ϵ parameter', whose value depends principally on the solar wind field direction, being maximum for southward fields and zero for northwards. Substorms are postulated to begin whenever ϵ exceeds a certain critical value such that an instability occurs in the magnetosphere, and to be continuously maintained so long as ϵ remains above this value. Akasofu suggests that the instability occurs in the current system connecting the magnetic tail with the nightside auroral ionosphere.

One important difference between the 'growth phase' and the 'direct' models is the expected magnetospheric response to intervals of steady southward solar wind magnetic fields. The 'direct' model would predict steady substorm-like conditions, while the 'growth phase' model suggests that a sequence of substorms would occur, whose time scale is determined by magnetospheric time constants. Another important difference concerns the expected behaviour of the tail field at substorm onset. In the 'direct' model the tail field may indeed often increase in strength before substorm onset, due to increasing ϵ and increasing solar wind ionosphere coupling. However, onset is not associated with dissipation of the increased tail energy as in the 'growth phase' model. Rather, since ϵ will generally continue to increase as it passes the critical value the tail field strength may also be expected to continue to increase. Thus in the 'direct' model the tail field is expected to develop and decay in concert with ϵ and with the expansion and recovery of substorms, while in the 'growth phase' model the tail field is expected to grow before the substorm and generally to decay after the onset.

Recently, Baker *et al.*³ have assembled a variety of observations of a large isolated substorm whose expansion phase onset occurred at about 0100 UT on 29 December 1976, which bears directly on the above question. The nightside magnetosphere was monitored by two spacecraft, one directly measuring the tail magnetic field at a distance of $\sim 30R_E$, the other detecting the changes in the energetic charged particle environment at $\sim 6.6 R_E$ which directly result from the growth and decay of the tail field. These data show that for ~ 1.5 hours before the substorm (determined from ground magnetic records) the tail field continuously increased in strength. In itself this result is compatible with both substorm models — it can be regarded as a good example of a 'growth phase' or, in terms of the 'direct' model, it can be argued

that ϵ was increasing during this period but remained below the critical value required for substorm onset. Unfortunately, solar wind magnetic field data are not available during this interval to test this interpretation. The critical observation, however, is that shortly after substorm onset the tail field started to decline, reaching initial values ~ 40 min after onset. This result argues strongly in favour of the 'growth phase' model in which stored tail energy is dissipated after onset.

An important objection to this conclusion may be made by asserting that while the tail field was increasing, substorms could indeed have been occurring (with ϵ above critical value), but at sufficiently high latitude that they were not observed at standard auroral zone observatories (about 65° – 70° magnetic latitude). Baker *et al.* rule out this possibility by examining data obtained from a widespread network of riometers. The riometers indicate when energetic electrons are precipitating into the upper atmosphere by measuring the attenuation of cosmic radio noise at 20–40 MHz resulting from the production of D region ionization. They gave no indication of substorm-associated precipitation for at least 7 hours before the observed substorm onset. The result is confirmed by a 'snapshot' taken of the aurorae ~ 10 min before substorm onset near the height of the growth of the tail field by a US Air

Force DMSP weather satellite, which shows the presence of quiet nightside auroral arcs, but no dynamic substorm features such as are plainly evident in the subsequent 'snapshot' taken by the same spacecraft ~ 100 min (one orbit) later.

Taken together, the data presented by Baker *et al.* clearly argue strongly for the 'growth phase' model of substorm onset. One feature of the data does, however, strike a somewhat discordant note. It is found that while the decline in the tail field is essentially complete ~ 40 min after substorm onset, the ground data and DMSP observations clearly indicate that intense substorm activity continued for at least another hour. This behaviour is reminiscent of Akasofu's 'direct' model, although the suggestion cannot be directly tested in this case due to the lack of simultaneous solar wind magnetic field data. It seems reasonable to suggest that, in future, thought might be given to an intermediate substorm picture in which the expansion onset is as described by the 'growth phase' model, as demonstrated by Baker *et al.*'s results, but where substorm-like conditions may be maintained after the stored energy has been largely dissipated by a sufficiently large direct energy input from the solar wind. □

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T cell function: Ly phenotype and the MHC

from Elizabeth Simpson

THE lymphocytes of the immune system comprise a heterogeneous collection of subpopulations of cells performing different functions. The main distinction is between B cells, which produce antibodies and can be recognized by the antibody molecules on their surfaces; and T cells, which subserve almost all the other functions of the immune system and can be distinguished from B cells by the Thy-1 antigen on their surfaces. T cells themselves, however, are functionally heterogeneous, and cell surface markers reliably identifying different functional subsets have been more difficult to find.

One of the earliest T lymphocyte marker systems to be described was that of the Ly markers, Ly 1, Ly 2 and Ly 3, first described by Boyse *et al.*¹. The importance of these markers lay in their differential expression on T cell subsets^{2,3} and it was soon shown that they could be used to distinguish between helper T cells (Ly 1⁺ 2⁻) and cytotoxic T cells (Ly 1⁺ 2⁺)^{4,5}.

At about the same time it became evident that T cells used self major histocompatibility (MHC) antigens as guides during

recognition of extrinsic antigens⁶. For cytotoxic T cells, the self-restricting or guiding elements were class I MHC antigens: H-2 K and D in the mouse, HLA-B and -C in man, whilst for helper cells the appropriate self determinants were class II MHC antigens: H-2 I in mouse, HLA-D in man⁷.

Thus two sets of correlations seemed to be apparent: first, cytotoxic T cell function was attributable to Ly 1⁺ 2⁺ cells and directed towards class I and class I-associated antigens. Second, helper cells were Ly 1⁺ 2⁻ and directed towards class II or class II-associated antigens. As a result of some recent experiments by Swain, however, the correlation between help and class II antigens and cytotoxicity and class I antigens has now broken down. Instead, she proposes that Ly phenotype correlates primarily with the class of MHC antigen rather than with function. The basis for this proposition is that, under certain con-

S. W. H. Cowley is a SERC Advanced Fellow in the Blackett Laboratory, Imperial College, Prince Consort Road, London SW7 2BZ.

Elizabeth Simpson is in the Transplantation Biology section of the Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ.

ditions, (1) T help can be found in populations of T cells responding to class I allo-H-2 antigens, and this help is mediated by Ly 1⁺ 2⁺ cells; and (2) cytotoxic T cells directed towards class II allo-H-2 antigens can have the Ly 1⁺ 2⁺ phenotype. This analysis was made using monoclonal anti-Ly 2 antibodies so that the ambiguities inherent in results from polyclonal antisera were eliminated.

It had previously been reported that Ly 2 antibodies would block killing by cytotoxic T cells. Swain therefore added anti-Ly 2 antibody to cultures in which T cell help was generated by an allogeneic response against allo-MHC antigens, either class I alone, class II alone or class I plus class II. The induction of helper cells against class I antigen alone was blocked by anti-Ly 2 antibody. In all other cases, anti-Ly 2 antibody failed to block help. This result is concordant with a previous report¹⁰ that helper T cells stimulated with class I allo-MHC antigens had the phenotype Ly 1⁺ 2⁺. In a second series of experiments Swain tested the effect of anti-Ly 2 antibodies on cytotoxic cells generated against either class I plus class II, class I alone or class II alone MHC alloantigens. Cytotoxicity directed against class I alloantigens was inhibited whilst that against class II alloantigens was unaffected. This finding is consistent with recent results of both Glasebrook and Pierres¹¹, using cytotoxic T cell clones directed against class II MHC antigens. These clones were phenotypically Ly 1⁺ 2⁺ or Ly 1⁺ 2⁺ (Swain's bulk culture activity was mediated by Ly 1⁺ 2⁺ cells: cells with a reduced expression of Ly 2 surface antigen) but the cytotoxic activity of these cloned lines could not be abrogated by Ly 2 antiserum in the assay. Swain's conclusions are that T cells directed towards class II MHC antigens express Ly 1 and (at low levels) Ly 2 and can have helper or cytotoxic function neither of which is blocked by anti-Ly 2 antibody. However the presence of at least some Ly 2 antigen on the surface of the cytotoxic cells distinguishes them from the normal Ly 1⁺ 2⁺ helper cells generated in response to class II antigens. She also concludes that the helper cells directed against class I antigens have a Ly 1⁺ 2⁺ phenotype and can be blocked by anti-Ly 2 antibody. This last conclusion, however, is perhaps open to one objection: it is possible that the helper activity could be mediated by cytotoxic cells making lymphokines — in particular, interleukin-2

which could substitute for helper T cells in the culture. This possibility is suggested by the recent discovery of cytotoxic T cell clones directed against class I antigens and producing interleukin-2^{11,12}. It is also in line with recent evidence that T cell clones with either helper or cytotoxic function can elicit delayed-type hypersensitivity — a T cell-mediated response — when injected

into the footpad of a mouse^{13,14}.

Nevertheless the unifying hypothesis put forward by Swain, that the Ly phenotype of a T cell and its MHC guiding antigen are the primary correlates, while function depends on the nature of the stimulation, is an attractive one, which puts into perspective a certain amount of apparently conflicting data recently accumulated. □

Great voids in the Universe

from Joseph Silk

ONCE astronomers were content to point to the distribution of galaxies in the sky and infer that the Universe is relatively uniform in appearance. More recently, the cosmic microwave background radiation, which samples the Universe at great depth, has provided dramatic confirmation of this homogeneity. It is uniform to at least three-hundredths of a per cent, apart from the dipole anisotropy over 180 degrees that is associated with the motion of our Galaxy. Our local region of space is obviously inhomogeneous, but this can easily be accounted for because the microwave background radiation is seen as being emitted from a very early phase in the history of the cosmos before structure fully developed. One can think of this epoch as corresponding to a cosmic 'surface' or photosphere where the radiation was last scattered, somewhat analogous to the photosphere layer of the Sun below which we cannot see. Before this moment of last scattering, which also coincides with the epoch of 'decoupling' of matter and radiation, the radiation was strongly scattered by ionized particles. As the Universe expanded, the electrons and protons cooled eventually to combine into hydrogen atoms, which are highly transparent to the radiation, and little scattering subsequently occurred.

Since then, small density fluctuations have grown, unimpeded by the drag of the radiation and driven by their own slight increase in gravitational attraction, and have gradually accreted more and more surrounding matter. Such primordial fluctuations eventually formed galaxies, clusters and even superclusters of galaxies. The smoothness of the radiation provides a measure of the degree of inhomogeneity of the Universe at the decoupling epoch, and a strong constraint on theories of galaxy formation.

The larger the structure that is observed, the greater is the challenge to any theory for the origin of large-scale structure. The recent discovery of a great void extending over some 10⁶ cubic megaparsecs in the

direction of the constellation Boötes has therefore attracted considerable attention (R.P. Kirshner, A. Oemler, P.L. Schechter and S.A. Schectman *Astrophys. J. Lett.* **248**, L57; 1981). Several smaller regions containing no luminous galaxies were discovered during the course of galaxy surveys in the vicinities of great clusters, but the Boötes void is considerably greater than these regions. It had previously been noted that galaxies surrounding the Coma cluster formed a great supercluster. But if galaxies were sprinkled at random locations in space, many of them should have possessed redshifts appropriate to distances intermediate between the Coma cluster and the Virgo supercluster, of which our own Galaxy is an outlying member. Instead, great gaps were found in the redshift distribution, and as redshift is a direct measure of distance, this was indicative of an almost complete absence of bright galaxies in the vast space between Coma and Virgo.

The void in Boötes was discovered by looking deep into three small regions of sky separated by up to 30 degrees, and measuring the redshifts (and hence the distances) of all galaxies brighter than 16th magnitude. In this way, a region of the Universe extending well beyond the Coma cluster was probed. When Kirshner and his colleagues analysed the data in each of the three windows, they found a surprising coincidence — there was a similar gap in the redshift distribution in all three data sets. In effect, they had surveyed three cone-shaped regions that passed through the same great hole in the distribution of galaxies. The only plausible explanation was that the width of the hole spanned at least the 30 degrees they had sampled, and this led to the estimate that it encompassed a roughly cubical volume some 100 Mpc on the side.

The absence of bright galaxies in such a volume can be interpreted in several ways. Perhaps the region is truly devoid of matter, and is a genuine void in space. Galaxy clustering permits the formation of holes, just as in some regions, great aggregations develop. This explanation runs into one possible difficulty, since small inhomogeneities should have been

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Joseph Silk is Professor of Astronomy at the University of California, Berkeley, California 94720.

present in the matter at the epoch of last scattering of the radiation. The inferred amplitude of the associated radiation fluctuations from which this hole and other similar structures grew must have been at least one part in 10^3 , and the fluctuations would extend over an angle of about 2° . One would expect to see traces of these fluctuations in the microwave background radiation; however recent observations suggest that they are probably absent. These primordial fluctuations could, in principle, be smoothed out if the intergalactic medium stayed ionized because of heating from some unknown process, which would rescatter the radiation. However, the scale on which such smoothing effects could operate is limited by the distance travelled by light at a time when the density was high enough to permit rescattering of the radiation and amounts to few degrees. If the implied radiation fluctuations are definitely not seen, the great void in Boötes cannot be a true hole but must contain matter in some form.

This leads to the intriguing implication that there is an underlying dark component of the matter distribution. One could imagine it to be distributed uniformly on scales of tens of megaparsecs. Only a modest fraction need be clumped to account for the galaxy haloes and clusters which most astronomers believe are bound by such dark matter. It is a sobering reflection on our role in the Universe that most of its contents are hidden from view. The nature of the pervasive dark matter may, however, soon be resolved. Two experiments tentatively reported in 1980 that the neutrino has a finite rest mass. Neutrinos are predicted by the big bang theory to fill space in numbers comparable with those of the photons of the microwave background, and several experiments are underway to confirm this result. If the neutrino mass ultimately is found to exceed even one-millionth of an electron mass, neutrinos would dominate the mass density of the Universe.

Why should the voids contain neutrinos but lack the atoms from which galaxies formed? Adjacent to the voids are huge superclusters of galaxies. Evidently galaxies accumulated in great sheet-like or filamentary structures, occupying a small fraction of the volume of space. The galaxies could not have simply participated in the collapse of the superclusters, otherwise they would have acquired extremely high random velocities that would grossly violate observational constraints. A more plausible possibility is that the matter originally collapsed while still in gaseous form into great sheet-like condensations that subsequently fragmented into galaxies. Gaseous matter can radiate away the kinetic energy acquired during free-fall collapse, and so the newly formed galaxies need not develop large random motions. This idea is the basis of the pancake (or blini) theory of

galaxy formation advanced by Ya. B. Zel'dovich and his colleagues in Moscow. A serious but not necessarily fatal objection to the blini theory is that all galaxies are predicted to form at a relatively late epoch. Astronomers have been searching for the luminous protogalaxies predicted by this scenario, but have had little success.

A final possibility is that the galaxy formation process has somehow been modified or even suppressed in the great

voids between the luminous galaxies. Perhaps the voids simply lack bright galaxies but contain vast numbers of faint, low luminosity galaxies or, alternatively, enormous amounts of very diffuse hot gas that has failed to condense into galaxies. Existing searches have not been sufficiently sensitive to test either of these hypotheses. One can only conclude, as with so many other astronomical problems, that improved observations are urgently needed. □

How the malarial parasite enters the red blood cell

from R.J. Wilson

MALARIOLOGISTS have been thrown into a state of excitement by the expectation that the question of how the malarial parasite interacts with and deforms the erythrocyte membrane in order to gain entry may soon be answered. The excitement stems from the rapid accumulation of information about the composition and structure of the erythrocyte membrane and its associated cytoskeleton, as well as the experimental identification of separate stages in the parasite's (merozoite) invasion sequence. The stages can be categorized simply as recognition, attachment, junction formation and entry.

Recognition

Both the resistance and the susceptibility of animal species to particular malarial parasites correlate with surface interactions between the parasite and the host red cell. More specifically, Miller and his colleagues have shown there is an association between 'receptors' for malarial parasites and red cell surface antigens¹. Human erythrocytes lacking the Duffy blood group antigens are resistant to invasion by merozoites of the simian parasite *Plasmodium knowlesi* — an observation which may explain the striking absence of *P. vivax* (a human malarial parasite) in West Africa where most of the population is Duffy negative. Unfortunately, continuous cultivation of *P. vivax* has not been accomplished *in vitro*, so it is not possible to test this hypothesis directly. Nevertheless, much data has been gathered to support the hypothesis, culminating in the observation that although *P. knowlesi* is, after all, able to recognize and attach to Duffy-negative human red cells, it is not able to enter them. It is the internalization process rather than recognition of an appropriate red cell that is defective.

A good case can be made that on human red cells glycophorin acts as a receptor for

the major human malarial parasite *P. falciparum*². Erythrocytes (so-called En(a-) cells) deficient in glycophorin A, the main sialoglycoprotein of the human red cell membrane, are less susceptible to parasite invasion *in vitro*. Furthermore, antibodies to glycophorin A block invasion, and the addition of purified glycophorin A to cultures can inhibit parasite invasion *in vitro*. Erythrocytes deficient in glycophorin B (S-s-u cells), which are relatively resistant to invasion, become almost completely resistant when trypsinized so as to remove the oligosaccharide-rich N-terminal region of glycophorin A. Interestingly, merozoites of *P. falciparum* are unable to distinguish between erythrocytes bearing MM, NN or MN antigens. As these antigenic structures are determined by the configuration of the five N-terminal amino acids of glycophorin A and the three associated oligosaccharides, the terminal region of glycophorin A seems not to be specifically involved in recognition and attachment.

Attachment

Adhesion of a merozoite to an appropriate erythrocyte has no immediate consequences unless the merozoite is oriented with its apical end (which contains various micro-organelles) to the erythrocyte membrane. This orientation results in a rapid spasmodic 'flinching' movement involving as much as one-third of the erythrocyte membrane³. The membrane then appears to relax with the merozoite still attached but largely external to it. Next, the parasite is drawn relatively slowly into a tightly localized invagination of the host cell membrane. After entry, sealing of the pore is accompanied by irregular but widespread movements of the erythrocyte membrane and slight swelling of the host cell.

The changes involved in this dynamic process take about 20 seconds to complete and can be deciphered by drawing on the static structural evidence of transmission electron microscope and freeze-fracture

R.J. Wilson is at the National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA.

studies, as well as models of the structure of the erythrocyte membrane and its associated cytoskeleton. Transmission electron micrographs indicate that the plasma membranes of the host erythrocyte and parasite do not fuse but the merozoite seems to be attached by its apical tip to the red cell membrane and contents discharged from the merozoite's rhoptry organelles may be implicated in adhesion.

Junction formation

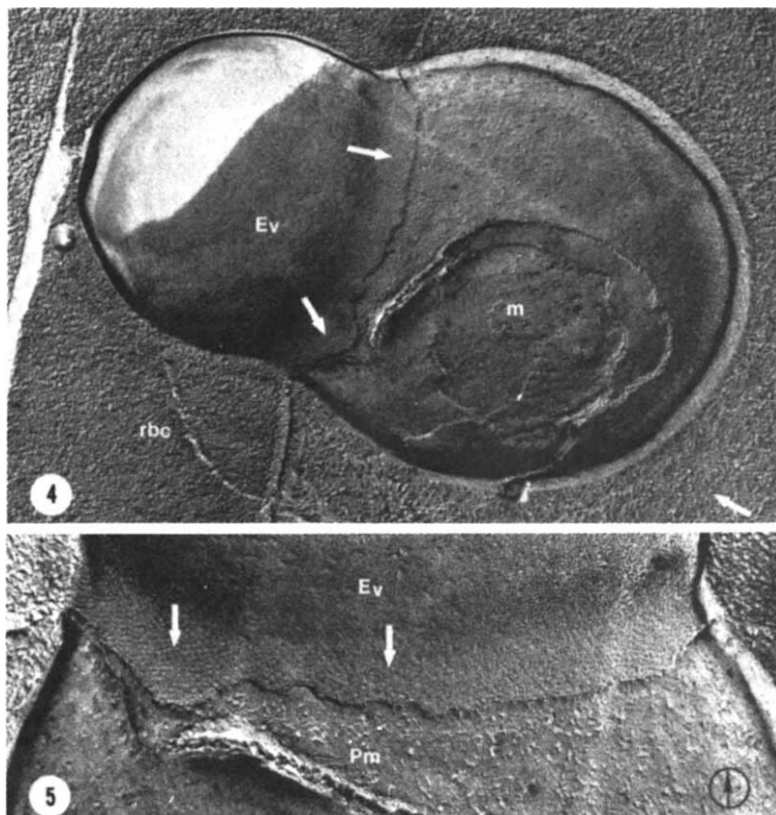
The subsequent invasion process is characterized by the development of a discrete junctional zone — an electron-dense thickening of the erythrocyte membrane closely applied (10 nm) to the parasite's surface coat in the apical region⁴. Electron micrographs indicate that the inner leaflet of the red cell membrane bilayer is thickened and Aikawa and his colleagues⁵ have demonstrated by freeze-fracture that in this zone in the protoplasmic or P-face of the membrane, there is an array of tightly packed intramembranous particles (IMPs). Both this and an earlier freeze-fracture study by McLaren *et al.*⁶ concur in describing IMP-free vesicles (or sections through a tortuous membranous channel) extending from the parasite's point of attachment deep into the erythrocyte's interior. All the changes just described still occur when merozoites treated with cytochalasin B interact with erythrocytes, even though the merozoites cannot complete the invasion process. Clearly attachment, which may involve recognition sites (receptors) and the formation of an IMP-rich junctional area of host cell membrane, needs to be followed by further steps to allow invasion.

One of these is the translocation of the junctional zone as a narrow ring-like structure along the longitudinal axis of the merozoite until it encloses it and forms part of the parasitophorous vacuole surrounding the internalized parasite. Aikawa *et al.*⁵ have confirmed the earlier important observation by McLaren *et al.* that the P-face of the invaginated membrane, which ultimately forms the parasitophorous vacuole, has a grossly reduced number of IMPs compared with the intact membrane. Unlike Aikawa, McLaren *et al.* reported few particles in the external or E-face of the invaginated membrane.

Entry into the red cell

During internalization, the surface coat of the merozoite is modified and can no longer be detected within the invagination by electron microscopy. Earlier suggestions that the surface coat accumulates outside the invagination and is eventually sloughed off have been challenged. Aikawa *et al.* maintain that the surface coat is unaltered external to the invagination and that a process akin to capping cannot be invoked to explain the parasite's internalization.

The motive forces responsible for invagination of the parasitophorous vacuole membrane and internalization of



Above, freeze-fracture electron micrograph of a merozoite(m) entering a red blood cell(rbc). The arrows indicate the E-face of the erythrocyte membrane. Ev is the E-face of the vacuole membrane. x37,000. Below, high-power electron micrograph of the neck of the invagination. The E-face of the vacuole (Ev) shows few IMPs. Pm, P-face of the merozoite membrane. x68,700. (From ref. 5.)

the attached parasite can only be guessed. Membrane flow past the junctional zone might cause its apparent lateral displacement. The formation of an IMP-rich patch around the initial attachment site and the subsequent invagination of a large IMP-depleted vacuole (commensurate with about 3 per cent of the erythrocyte's surface area) suggest that peripheral membrane proteins such as spectrin and actin are dissociated from the cytosol surface of the erythrocyte membrane to allow lateral redistribution of IMPs. In intact erythrocytes there is evidence that IMPs represent the integral membrane proteins, band 3 and glycophorin. These have restricted lateral movement because of interaction with the cytoskeletal network⁷. It is still unclear whether a histidine-rich (73 per cent) parasite protein is released during invasion and acts like other polyanionic substances to disrupt the cytoskeletal network⁸, and whether the IMPs aggregated in the junctional zone by the malarial parasite actually correspond to erythrocyte proteins.

So far there have been few studies of the invasion of erythrocytes with modified cytoskeletons. Recently, it was reported that ovalocytic erythrocytes from Melanesians are resistant to invasion by *P. falciparum*⁹. This could be due to an alteration in the erythrocyte's cytoskeleton that reduces the physical deformability of the membrane or prevents the

redistribution of IMPs required for junction formation. Alternatively, there may be a defect in the distribution of surface receptor sites. Access to the cytoskeleton for specific reactions with antibodies and other reagents has been achieved by the formation of resealed erythrocyte ghosts which retain their susceptibility to parasite invasion¹⁰. Preliminary studies (Dluzewski, personal communication) with resealed erythrocyte ghosts containing antibodies to spectrin indicate that parasite invasion is markedly reduced. This approach promises to help clarify the rearrangement of peripheral and integral erythrocyte membrane proteins during invasion. Clearly, many challenging problems remain to be solved at the molecular level before we can claim to understand how the major haemotropic pathogen of man makes its temporary escape from the extracellular environment by invading red cells. □

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The mystery of the mouse α -globin pseudogene

from R.A. Flavell

THE chromosomes of multicellular organisms seem to be full of a wide variety of DNA sequences which are of no apparent use to their hosts. Some of these sequences, termed pseudogenes, are defective copies of functional genes. The laboratory mouse has two such crippled genes — the α -globin pseudogenes. However, one of them, $\alpha\psi 3$, is different from other pseudogenes described to date. It has precisely (to the nucleotide) lost the intervening sequences (or introns) which are present in the functional α -globin genes^{1,2}. It is not known why introns are commonly found in the genes of vertebrates — they are removed from the primary RNA transcript during production of a functional messenger RNA and it seems a complicated method of encoding genetic information when intronless genes work well in prokaryotes (for speculations on this subject see ref.3). But whatever the reasons why the insertions are present in the normal α -globin genes; the question of how $\alpha\psi 3$ lost its introns is a riddle of great interest.

One possible origin of $\alpha\psi 3$ is that an intronless α -globin mRNA altered a duplicated normal α -globin gene to an exact copy of itself by gene conversion¹. However, although gene conversion between homologous DNA sequences is well characterized, gene conversion by a RNA has not been seen. Another, perhaps more plausible possibility is that $\alpha\psi 3$ somehow derived from the copying of an α -globin mRNA into a DNA molecule by the retrovirus-encoded enzyme reverse transcriptase. Such a DNA might also alter a duplicate α -globin gene by gene conversion.

The most exciting proposal is, however, that an α -globin mRNA actually became incorporated into the RNA genome of a retrovirus. Retroviruses are equally at home as either RNA-containing viruses or DNA copies of this RNA (called proviruses) which are integrated into the host cell DNA. Therefore, provided that one accepts that an α -globin mRNA could gain entry into the retrovirus genome, it is only necessary to invoke the normal infectious cycle of the virus to explain the presence of $\alpha\psi 3$. Retroviruses are certainly capable of incorporating cellular genes into their RNA genomes⁴ and, significantly, comparisons between the cellular and viral genes show that the RNA copies of these cellular genes have sometimes lost the introns which characterize the parent genes⁵. So far the only genes known to have been 'captured' by a retrovirus are those conferring an easily scored phenotype on infected cells (tumorigenic

transformation), but it is reasonable to suppose that a retrovirus might incorporate any piece of the cells' transcribed DNA, such as the α -globin gene. Of course, such an event would have to occur in the germ line of the mouse to survive the eventual death of its host.

Such a model demands that the newly integrated α -globin gene be accompanied by a retrovirus provirus. In this issue of *Nature* (p.426), Lueders *et al.* describe their analysis of the flanking DNA of both α -globin pseudogenes and the α -globin genes of mouse. They show that $\alpha\psi 3$ is indeed flanked by a pair of retrovirus-like elements whereas the other α -globin genes are not. The elements, which are present in about 1,000 copies per mouse genome, encode a class of virus-like entities called intracisternal type A particles (IAPs) which share close similarities with the commonly studied type B and type C retroviruses. Unfortunately, it is by no means clear whether IAP elements are responsible for the creation of $\alpha\psi 3$, as the pseudogene is flanked on either side by about 4 kilobases of cellular DNA before the IAP elements are reached, whereas the model demands that $\alpha\psi 3$ be actually connected to the retroviral sequences. It is at this point that the real problem in determining the origin of this pseudogene raises its head.

The pseudogene emerged roughly 20 million years ago, since when there may have been numerous alterations to the gene

and its immediate environment, perhaps because $\alpha\psi 3$ was probably superfluous to the metabolic requirements of its host. Deletion or insertion of other DNA sequences may have masked its original configuration. The authors point out that a way around this problem may be to study the same pseudogene in related mouse species and in the rat. By looking for alterations which have cropped up during the evolution of these species, the original configuration of $\alpha\psi 3$ may be revealed. Two of the authors are accumulating more data on mouse genes flanked by other IAP elements which should show whether the $\alpha\psi 3$ -IAP complex is particularly unusual. Doubtless, the sequences between the pseudogene and the IAP elements are also being scrutinized for clues to the origin of the arrangement.

If IAPs do turn out to have a role in the creation of the α -globin pseudogene, could such a phenomenon affect genome evolution by inducing the migration of functional DNA sequences to new sites? Or are we looking at a way of creating merely new 'junk' DNA? □

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100 years ago

THE INDIAN DARTER

The Darters form a very peculiar type of birds of the order Steganopodes, allied to the Cormorants in structure, but very Heron-like in gait and gesture. The Darter in its normal position sits erect upon a branch or stump overlooking the water. When proceeding to fish it dives head foremost into the stream, and swimming entirely under water, transfixes its



The Indian Darter

finny prey with the rapidity of lightning. Emerging from the water with the fish speared upon its long slender beak, the Darter chucks the fish into the air, and catching it head foremost with unerring aim, swallows it whole.

The Darters usually exhibited in the Royal Society's Gardens are of the South American species (*Plotus anHINGA*), but in 1878 an example of the African form Le Vaillant's Darter (*Plotus levaillanti*)¹ was received. In April last an example of a third species of this genus — the Indian Darter (*Plotus melangaster*) was obtained in exchange from the Zoological Gardens of Calcutta.

"In a state of nature," as Dr. Jerden tells us, "this beautiful diver is found throughout all India, Ceylon, Burmah, and Malayana. It is exceedingly numerous in some parts of the country, especially in Bengal; hundreds are often to be seen on a single jheel. They hunt singly in general, or in scattered parties, but often roost in company, both at night and in the middle of the day, when numbers may be seen perched on the trees overhanging some tank or river. They float low on the water, often with nothing but the head and neck visible, and swim and dive with rapidity. After feeding for some time they perch on the bough of a tree or on a pole or stone, and spread their wings out to dry as the Cormorants do." From *Nature* **25**, 24 January, 297, 1882.

R.A. Flavell is at the Imperial Cancer Research Fund Laboratories, Burton Hole Lane, London NW7 1AD.

REVIEW ARTICLE

Grain alignment in the galactic magnetic field

Paul E. Johnson

Wyoming Infrared Observatory, Department of Physics and Astronomy, University of Wyoming, Laramie, Wyoming 82071, USA

The linear polarization of light observed in many distant stars, presumably an effect of absorption of starlight by elongated interstellar dust grains, provides a means of mapping the magnetic field lines in our own Galaxy as well as probing the properties of the intervening dust. Processes for grain alignment in the galactic magnetic field are reviewed: evidence is examined for grains being spun up to extremely high (10^9 Hz) angular frequencies by the recoil of hydrogen recombination on grains, as an essential part of the alignment process. Grain alignment would then be inhibited in regions of grain growth and would be most effective where grain growth is inhibited or reaches saturation.

WHEN the light from stars close to the galactic plane is analysed, it is found to be both reddened and polarized, with the angle of polarization most often lying in the galactic plane. Both are presumed to be effects of absorption of starlight by a medium containing clouds of elongated dust grains (accompanied by neutral hydrogen), spinning so that their major axes tend to be transverse to the plane of the galaxy. The starlight transmitted through such a medium would appear reddened and linearly polarized in a direction parallel to the galactic plane.

The typical interstellar grain is usually found in diffuse clouds composed of a relatively high density of dust ($\sim 8 \times 10^{-11}$ grains cm^{-3}) and neutral hydrogen gas (~ 20 atoms cm^{-3})¹. Inside such denser clouds grains are shielded from the destructive effects of stellar UV radiation and can therefore grow by the accretion of volatiles such as H_2O , CH_4 and NH_3 . Grain cores are expected to be composed of more refractory materials, such as silicates, SiC and graphite. The observed optical properties of grains indicate overall grain diameters of 0.02 to 0.15 μm (ref. 2).

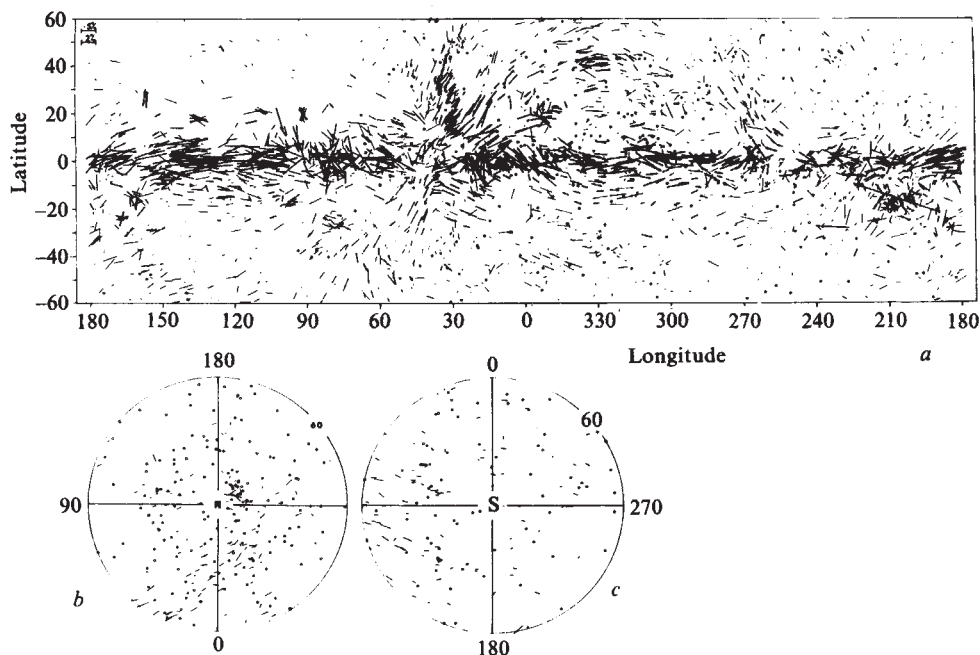
Martin³ pointed out that a rotating, charged grain will precess about a magnetic field, regardless of the mechanism aligning the grain. The result of such a precession is that, on a time average,

the distribution of grain axes will be symmetric about the magnetic field, if the period of precession is adequately short. Dolginov and Mytrophanov⁴ suggested that the Barnett effect, the spontaneous alignment of atomic dipoles in a rotating body, will produce a rapid precession in a magnetic field, even for an uncharged grain. For physical conditions in the interstellar medium the period of either type of precession is much shorter than the time constant for any previously proposed alignment mechanism. Therefore, the polarization of starlight should either be parallel or perpendicular to the magnetic field.

Figure 1 shows that the most orderly regions of polarization are those near the galactic plane exhibiting a polarization parallel to that plane. Therefore, it is reasonable to conclude that the general galactic magnetic field probably runs either parallel or perpendicular to the galactic plane⁵.

The increase in the amount of Faraday rotation (which gives $n_e B_{\parallel}$ integrated along the line of sight, where n_e is the electron number density in the interstellar medium and $B_{\parallel}$ is the component of the magnetic field directed along the line of sight) with decrease in galactic latitude, as measured in several hundred extragalactic radio sources, is consistent with radiative transfer through a general galactic magnetic field⁶. Uncertain-

Fig. 1 Interstellar polarization. The direction and degree of linear polarization of nearly 7,000 stars is shown with respect to galactic coordinates. The length of each line is proportional to the linear polarization. Stars with a polarization $< 0.08\%$ are represented by small circles. (After ref. 5. With the permission of the Royal Astronomical Society.)



ties in the electron density and variations in the magnetic field, B , over long pathlengths prevent an accurate determination of the galactic magnetic field from these data¹. Measurement of the dispersion of pulsar signals (which gives n_e integrated along the line of sight) and their Faraday rotation allows an accurate determination of both the electron density and magnetic field in the solar neighbourhood. The uniform component of the magnetic field in the solar neighbourhood has been measured at $2.2 \pm 0.4 \times 10^{-6}$ G in this way⁷. The variation of the magnetic field with galactic longitude indicates that B runs roughly parallel to the galactic plane. The polarization of starlight has been one of the chief tools with which to investigate the large-scale structure of the galactic magnetic field. I review here the two most viable theories of grain alignment and assess the evidence bearing on them.

Davis–Greenstein grain alignment

It has been generally assumed that interstellar grains are aligned with respect to the interstellar magnetic field by paramagnetic relaxation of thermally rotating grains, as originally proposed by Davis and Greenstein⁸ (DG alignment). In this process a paramagnetic grain is set spinning by impacts with the interstellar gas. When such a grain is immersed in an external magnetic field an internal field is induced such that its direction is coincident with the external field. It is impossible for the alignment of the internal and external fields to be achieved instantaneously. Because the grain is spinning there is always a slight misalignment between the two fields. The external field attempts to enforce alignment with a drag torque opposing the angular velocity of the grain about an axis perpendicular to the direction of the magnetic field. This results in grain alignment. DG alignment of aspherical particles was modelled by Purcell⁹ and Purcell and Spitzer¹⁰ using a Monte Carlo analysis of the entire process. Their analyses confirmed the theoretical results of Jones and Spitzer¹¹ that alignment of grains adequate to explain the interstellar polarization of starlight would require a magnetic field $> 10^{-5}$ G, much larger than what has been found empirically.

To achieve effective DG alignment, the collisional damping time of grains (due to gas–grain collisions) t_d , must be less than or equal to the paramagnetic relaxation time (the time scale for alignment) t_r (refs 10–12). In a H I cloud one must have

$$\delta \equiv \frac{t_d}{t_r} \approx \frac{B^2 \chi''}{\omega n_H m_H \bar{v}_H a} \leq 1 \quad (1)$$

where χ'' is the complex part of the magnetic susceptibility of the grain material, ω the angular frequency of the grain, n_H the number density of hydrogen atoms, $\bar{v}_H$ the r.m.s. velocity of hydrogen atoms, and a the mean radius of an interstellar grain. For the conditions in a 'typical' H I cloud (the neutral hydrogen density, $n_H = 20 \text{ cm}^{-3}$, $T_g = 80 \text{ K}$, $T_d = 15 \text{ K}$, and paramagnetic grains with $a = 0.3 \mu\text{m}$ (ref. 1)) B must be greater than 8×10^{-6} G. So large a magnetic field in H I clouds is inconsistent with the upper limit of 3×10^{-6} G from Zeeman measurements¹³.

This problem can be circumvented by assuming that the grains responsible for interstellar polarization are weakly ferromagnetic or superparamagnetic. The latter occurs when isolated clusters of iron atoms, less than $100 \text{ \AA}$ in size, behave as single, giant magnetic moments¹⁴. Duley¹⁵ has suggested the existence of weak ferromagnetism or superparamagnetism in grains of mixed MgO FeO SiO. According to this theory, if the cores ($100\text{--}200 \text{ \AA}$ in size) of $0.1\text{--}0.3 \mu\text{m}$ grains are composed of such material and covered with ice, then they can be aligned in a field of only 1×10^{-6} G. If the cores are weakly ferromagnetic, relaxation is caused by the spin-lattice relaxation of paramagnetic spins in a mantle of icy material. If, on the other hand, the grains are superparamagnetic, relaxation proceeds as in the case of paramagnetic relaxation, but with fields $< 1 \times 10^{-6}$ G. However, it is not certain that this latter process is effective in cores $100\text{--}200 \text{ \AA}$ in size.

In any case, such mixed oxide grains would reproduce the spectral resonances at $3.1 \mu\text{m}$ and $10 \mu\text{m}$ seen in such sources as the Becklin–Neugebauer Object (BN) in Orion¹⁶. Other ferromagnetic materials, such as magnetite¹⁷, used in explaining interstellar polarization cannot easily reproduce these spectral features.

Most other alignment mechanisms are greatly inferior to DG in explaining grain alignment in the galactic magnetic field¹⁴. For example, Gold's streaming mechanism (in which grains present their smallest cross-section to a stream of rapidly moving gas) falls short of DG by an order of magnitude in explaining the general interstellar polarization⁹.

Purcell pinwheel alignment

Purcell's^{18,19} pinwheel alignment mechanism seems to be viable for the physical conditions in the interstellar medium. In this theory, a grain spins-up to high angular velocities when inhomogeneities on the surface of the grain result in a mean torque with respect to an axis fixed in the grain. Such torques arise, for example, when hydrogen atoms collide with a grain and stick to it, diffusing over the surface of the grain until they combine with another hydrogen atom at an 'active' site. Hollenbach and Salpeter²⁰ suggest that a limited number of active sites exist on a grain and are simple impurities or surface defects that serve to catalyse the formation of molecular hydrogen. Such a molecule is subsequently ejected with a linear momentum p_0 , resulting in a relatively large torque being imparted to the grain. For ν active sites randomly distributed over the surface of a grain, the mean torque applied by such a collision is about $ap_0/\nu^{1/2}$. A torque can also be the result of a variation in the photoelectric emissivity or the accommodation coefficient (the fraction of gas atom kinetic energy transferred to the grain in a gas–grain collision) over the surface of a grain.

All of these processes will produce relatively strong systematic torques, leading to rotational frequencies as high as 10^9 s^{-1} , corresponding to a rotational velocity, (ωa) , of 10^4 cm s^{-1} for a grain of radius $0.1 \mu\text{m}$. In comparison, the mean velocity of a gas atom for $T_g = 80 \text{ K}$ is $1.3 \times 10^5 \text{ cm s}^{-1}$. Purcell¹⁹ shows that in the presence of dissipative processes, such as anisotropic elasticity, the grain's angular momentum direction will lie along the principal moment of inertia. Note that almost nothing is known about the shape of grains other than that elongated grains are required to generate the observed polarization of starlight.

Of the above processes, the hydrogen recombination pinwheel should be the most effective in the interstellar medium by several orders of magnitude where hydrogen is mainly in the atomic form.

Effect of grain resurfacing on alignment

The Purcell pinwheel mechanism possesses quantitative difficulties in explaining grain alignment^{1,19,21}. These problems arise because we previously assumed that the net torque due to anisotropies on the surface of a grain does not change with time. Here only the case of hydrogen recombination driving the grains suprathermally, the strongest of the processes suggested by Purcell, will be considered, although the results are quite general. Both the theory of grain growth and the measurement of the increase of λ_{max} , the wavelength of maximum linear polarization, with distance into a dark cloud indicate that grains are growing¹. Therefore, over a period of time, t_L , old active sites will be covered up, leaving new ones. Whether or not the locations of the new sites are correlated with those of the old sites is unknown. It is reasonable to assume that the new torque on a grain would be entirely altered when the radius of a grain is increased by a single monomolecular layer.

Spitzer¹ gives the rate of increase of the radius of a grain due to the accretion of atoms, excluding H and the noble gases, with cosmic composition as

$$\frac{da}{dt} \approx 2 \times 10^{-13} \xi \text{ cm yr}^{-1} \quad (2)$$

where ξ is the effective sticking coefficient, $T_g = 80 \text{ K}$, and

$n_H = 20 \text{ cm}^{-3}$. Most growth in dust clouds should be due to the accretion of oxygen and the resulting formation of ice. In $2.4 \times 10^5 \text{ yr}$, the collisional damping time in these conditions, the radius of a grain changes by about $6 \times 10^{-8} \text{ cm}$, exceeding the thickness of a monomolecular layer.

Just as serious as grain growth is the constraint put on Purcell alignment by grain denudation due to UV radiation in unshielded nebulae as well as in dust clouds where there has been a burst of UV radiation during star formation. Such denudation would resurface grains just as effectively as grain growth.

Purcell¹⁹ and Spitzer and McGlynn²¹ treat the effect of the alteration of a grain surface on pinwheel alignment in some detail. Let us assume that after a time t_L the torque on a grain, in body coordinates, will have been completely altered. Unfortunately, the effectiveness of paramagnetic relaxation on grain alignment depends critically on t_L , which is uncertain at best. If $t_L \gg t_r$ grains will spin-up to an equilibrium angular velocity in a time t_d , and will then become aligned. However, if t_L is small, the torque about the principal moment of inertia will occasionally change sign and the angular velocity of the grain about the axis will 'cross-over' through zero. The extent to which alignment is affected depends on the magnitude of the change in grain orientation during cross-over and the cross-over frequency. Purcell¹⁹ shows that the mean time between cross-overs is t_x , where

$$t_x = 1.3(t_L + 0.6t_m) \quad (3)$$

t_m is the time required for a grain to collide with a mass of gas equal to its own mass. If $t_x \sim t_m$ and a grain loses all memory of its orientation during a cross-over, then the Purcell mechanism falls short by at least an order of magnitude in explaining the observed polarization of starlight.

Spitzer and McGlynn²¹ used a Monte-Carlo analysis to determine how much orientation a grain loses during cross-over. They were only able to strengthen marginally the ability of the Purcell mechanism to align grains. Some of the physical parameters affecting their model (such as grain size and density) are rather poorly known, however, so that the outcome is not certain.

There are at least two sets of conditions in which pinwheel alignment is still viable. If the grains are superparamagnetic or ferromagnetic χ'' can be larger by up to a factor of 10^5 (ref. 1). Then (t_m/t_r) is small enough so that appreciable alignment is likely, even with fields of 10^{-7} G . Another possibility is the inhibition of grain resurfacing.

Grain growth and destruction in the interstellar medium

Observations of the wavelength dependence of the polarization of the stars obscured by the R Coronae Australis dark cloud has led Vrba *et al.*²² to conclude that there is an evolution of grain size distribution with age and changing environment (Fig. 2). R CrA is a young cloud ($\leq 10^6 \text{ yr}$)²³ with its high density and low internal stellar luminosity favouring the formation of large grains. The large variation in grain size (as reflected in the large dispersion in λ_{max}) is likely to be caused by individual grains being formed and growing in different conditions in different parts of the cloud. Stars near the edge of the cloud, for example, would be less shielded than grains in the interior and would therefore experience a slower rate of growth and a smaller mean size.

The α Persei cluster (an analogue of the R CrA cloud with an age of $2 \times 10^7 \text{ yr}$) has a smaller mean grain size, close to that of the general interstellar medium²². Coyne *et al.*²⁴ show that in $\sim 10^7 \text{ yr}$ large grains can be destroyed by photodesorption during a phase of extremely luminous pre-main-sequence evolution, which the α Per cluster has apparently experienced and to which the R CrA cloud has not yet evolved. Stellar winds and radiation pressure may have dispersed large grains, and, of course, UV radiation would be expected to dissipate the mantles of grains, thus reducing their size.

We will assume that grains in the diffuse interstellar medium are cores denuded of their mantles and are strongly growth inhibited by UV desorption. Such grains, almost regardless of their composition, will experience efficient alignment by the Purcell pinwheel alignment process due to the non-existence of an efficient grain resurfacing mechanism in these conditions.

The rate at which an interstellar grain accretes material by gas-grain collisions is given by $\pi a^2 n_a \bar{v}_a \xi$, where n_a is the number density of atoms in the gas other than hydrogen and the noble gases and $\bar{v}_a$ is the r.m.s. velocity of these atoms. The rate at which atoms are desorbed from a grain is given by $\pi a^2 Y_{\text{pd}} G_0 \exp(-\tau_{\text{uv}})$, where Y_{pd} is the photodesorption yield of a grain, G_0 is the integrated diffuse interstellar photon flux between 912 (the Lyman limit) and $1,900 \text{ \AA}$ (longward of which photodesorption is rather ineffective²⁵), and τ_{uv} is the effective extinction in the UV to the region under consideration²⁵. One would expect that even if there is no net grain growth, accretion and photodesorption will redistribute active sites more rapidly

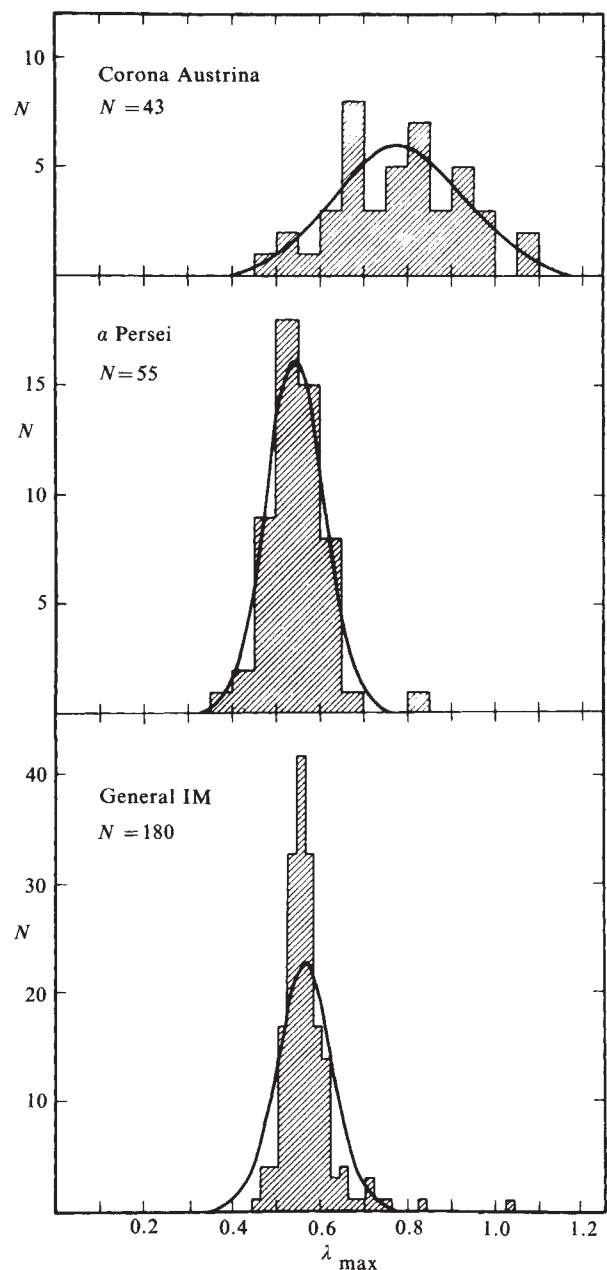


Fig. 2 Histograms of the grain size distribution (as measured by the wavelength of maximum polarization, λ_{max} , for an obscured source) for the diffuse interstellar medium (IM) and the α Per and R CrA dark clouds. The solid curves represent normal distributions fitted to the data. (After ref. 22. With the permission of the University of Chicago Press.)

than accretion alone. For the activation of the Purcell pinwheel mechanism by photodesorption it is then necessary that grains are completely stripped of volatiles, or

$$n_a \bar{v}_a \xi < Y_{pd} G_0 \exp(-\tau_{uv}) \quad (4)$$

In the interstellar medium nominally we have $\xi \approx 1$, $Y_{pd} \sim 5 \times 10^{-3}$, $G_0 \sim 1.7 \times 10^8 \text{ cm}^{-2} \text{ s}^{-1}$, and $\bar{v}_a \sim 8.0 \times 10^3 \text{ cm s}^{-1}$ (corresponding to the r.m.s. velocity of oxygen atoms at 80 K)²⁵. Therefore $n_a \exp(\tau_{uv}) \leq 8 \times 10^4 \text{ cm}^{-3}$ in order for grain resurfacing to be effectively halted²⁵. This criterion would in general be met in the diffuse interstellar medium, but not necessarily in the interiors of dense molecular clouds.

In the above model, grains shielded from photodesorption in the dense interiors of dark clouds would be less susceptible to alignment than unshielded grains. Indeed, it has been observed in both the ρ Ophiuchus dark cloud²⁶ and in R CrA (ref. 22) that the polarization efficiency of grains (as measured by p/A_v , where p is the linear polarization of an obscured object and A_v is its visual extinction) decreases dramatically with the mean size of grains in a particular region (as measured by $\lambda_{\max}$ and the ratio of total to selective extinction, R). This may be due to the anticorrelation between grain alignment and rate of growth in the Purcell model. Such results are also consistent with DG alignment, which is most effective with small particles.

The major difficulty with our model is the efficient grain alignment in regions of dense clouds having large $\lambda_{\max}$, such as the Orion Molecular Cloud (OMC-1) (ref. 27) and M17 (ref. 28). I suggest that the solution to this apparent dilemma is to dissociate large $\lambda_{\max}$ from a high growth rate. The rate at which the radii of grains can grow due to atomic accretion, in the absence of photodesorption, is given by equation (2). It then follows that the time scale for the adsorption of a molecule or atom by a grain is $5 \times 10^{15} a / T^{1/2} n_a \text{ yr}$ (refs 22 and 29), for a in cm, T in K, and n_a in cm^{-3} . The shortness of this time scale (of the order of 10^6 yr for a typical dark cloud) implies two things:

(1) In the regions of dark clouds adequately shielded from UV radiation, grain growth due to molecular accretion will rapidly saturate. The saturation of R in the ρ Oph cloud with $A_v \geq 3.5$ has been shown to be consistent with all heavy elements having been accreted onto grain mantles³⁰⁻³². The correlation between the column density of ^{13}CO versus A_v in the dark cloud L134 up to $A_v \sim 3-5$ can be interpreted as being caused by all C and O being deposited on grain mantles when $A_v \geq 4$ (refs 30, 33).

(2) Grain growth in dense clouds, as indicated by the increase in $\lambda_{\max}$ with extinction is probably caused by coagulation of individual grains and not by accretion of atoms and molecules onto grain surfaces³⁴. Accretion can only increase the mean grain size by a factor of ~ 2 , corresponding to the exhaustion of all condensable species in the gas³⁵.

In these conditions, I suggest that the time scale for grain-grain coagulation from systematic collisions between grains, when acted on by an external force such as gravity or radiation pressure^{22,29} is, the limit to spin-up (due to resurfacing) through the Purcell pinwheel alignment mechanism. This is given by

$$t_{\text{gg}} \approx 8 \times 10^{17} a / n_d V_d \text{ (yr)} \quad (5)$$

V_d is the drift velocity of grains with respect to the gas and n_d is the number density of dust grains. V_d , a and n_d are in cgs units. For the inner regions of OMC-1 we estimate t_{gg} (using the formulae in refs 22, 29) at $\sim 10^9 \text{ yr}$. This limit is weak enough to place no constraint whatsoever on Purcell alignment. For Purcell alignment to be effective the alignment time, t_r , must be significantly smaller than the time scale for resurfacing, t_{gg} in this case, or

$$\left(\frac{t_r}{t_{\text{gg}}} \right) = 4 \times 10^{-14} \frac{a \rho T_d n_d V_d}{B^2} \ll 1 \quad (6)$$

with ρ the grain density. For our typical H I cloud with $\rho = 1 \text{ g cm}^{-3}$, $n_d = 7.6 \times 10^{-11} \text{ cm}^{-3}$, and $B = 2.5 \times 10^{-6} \text{ G}$ we have

$t_r/t_{\text{gg}} \approx 4 \times 10^{-11} V_d$ which is much less than unity for any reasonable choice of V_d .

Grain alignment in star formation regions

Until recently there has been no empirical evidence for pinwheel alignment. Johnson *et al.*²⁷ have demonstrated the quantitative inadequacies of DG alignment in the OMC-1 quite independent of grain composition. The high density of the nebula means that the temperatures of the dust and molecular gas are expected to be tightly coupled. This has been seen in H_2CO maps³⁶ and 91- μm photometry³⁷ of the nebula (indicating the temperature of the gas and dust, respectively). DG alignment is driven by differences in the dust and gas temperatures and therefore cannot operate in a region where $T_d \approx T_g$ (ref. 38). Johnson *et al.* hypothesize that grain alignment in OMC-1 is due to pinwheeling, the only viable alternative to DG alignment. The atomic hydrogen abundance necessary to excite hydrogen recombination pinwheeling would be provided by atomic hydrogen left in the wake of a shock propagating through the nebula. Evidence for a shock in OMC-1 has been found³⁹ from a broad velocity feature ($\Delta v \approx 49 \text{ km s}^{-1}$) in CO and detection of H_2 vibrational emission. Alignment would occur in OMC-1 with a relatively weak (milligauss) magnetic field.

Dyck and Capps⁴⁰ have measured the IR linear polarization of 15 compact sources associated with H II regions and molecular clouds. They find a clear correlation such that strong interstellar polarization is associated with the youngest prestellar objects. Dyck and Capps hypothesize that as these objects evolve, the conditions for producing polarization can no longer be maintained. This is the situation that shock-induced grain alignment would predict. Strong shocks in molecular clouds, due to stellar winds accompanying star formation, would produce grain alignment (and thus linear polarization) in the same way that Johnson *et al.*²⁷ suggest for OMC-1. As prestellar objects evolve onto the main sequence and produce H II regions, shocks in the surrounding molecular cloud will dissipate resulting in the decay of linear polarization of sources embedded in the cloud as all of the atomic hydrogen is consumed. Another possibility is that as a number of luminous pre-main-sequence stars 'turn-on' in the dust cloud, resurfacing of grains by photodesorption begins, thus inhibiting grain alignment.

Conclusions

Both the Purcell alignment mechanism and DG alignment can quantitatively explain interstellar polarization. However, both theories have apparent drawbacks. DG alignment requires invoking ferromagnetic or superparamagnetic grains, thus placing rather stringent requirements on grain composition. DG alignment also seems to fail to explain grain alignment in OMC-1. Although the Purcell theory seems to be equally viable for almost any grain composition, it does require rather small grain resurfacing rates.

In any case, it seems that the typical interstellar grain is suprathermally rotating, and any grain alignment theory must deal with this. The Purcell mechanism would predict that grain alignment is most effective where: (1) grain growth has been inhibited either due to UV photodesorption or depletion of accretable material; and (2) there is a significant atomic hydrogen abundance or an adequate ambient radiation field.

These two conditions seem to be met not only in the unshielded regions exterior to dark clouds but also in regions where pre-main-sequence stars are undergoing a burst of luminosity. Significant progress can be made in this field by checking the predictions above against a detailed set of measurements of the polarization of sources found in or behind a number of clouds exhibiting a wide variety of conditions.

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ARTICLES

Ultrasonic borehole televiewer investigation of oceanic crustal layer 2A, Costa Rica Rift

Mark D. Zoback

United States Geological Survey, Menlo Park, California 94025, USA

Roger N. Anderson

Lamont-Doherty Geological Observatory, and Department of Geological Sciences, Columbia University, Palisades, New York 10964, USA

In situ lithostratigraphy and the distribution of fracture and void zones in oceanic layer 2A were examined with an ultrasonic borehole televiewer at IPOD sites 501 and 504B on the south flank of the Costa Rica Rift. These records indicate a decrease in the size of basalt pillows with depth and a corresponding increase in fracture and void density. The observed increase in P-wave velocity with depth in this hole, as layer 2A merges into 2B, is best explained by an increase in the degree to which clays and zeolites fill fractures and voids. This also explains the order-of-magnitude drop in permeability observed in hole 504B. An increase in alteration-filling with age could explain the transition from convective to conductive heat flow and the correlative disappearance of layer 2A on the flanks of oceanic spreading centres.

HEAT flow measurements on the flanks of mid-ocean ridges show a transition from primarily convective heat flow near ridge axes to predominantly conductive heat flow in old ocean basins¹. Coinciding with the change in heat transfer mechanism, oceanic crustal layer 2A essentially 'disappears' with age on the flanks of mid-ocean ridges (Fig. 1; ref. 2). Layer 2A is defined as a seismic transition zone in which a section with anomalously low P-wave velocity ($V_P \sim 2-3 \text{ km s}^{-1}$) overlies high-velocity layer 2 basalts ($V_P \geq 5 \text{ km s}^{-1}$). This transition occurs within the interlayered pillow, flow and rubble zones of the upper 1 km of oceanic crust. To investigate the interrelationship between these thermal and acoustic phenomena and other physical properties of the oceanic crust, we participated in an integrated suite of *in situ* downhole experiments at IPOD sites 501-504B on the south flank of the Costa Rica Rift of the Galapagos Spreading Centre (Fig. 2). The experiments included *in situ* permeability and pore-pressure measurements³, sonic velocity logging⁴ and formation fluid sampling⁵. Here we describe ultrasonic borehole televiewer imagery that was conducted to examine in detail the lithostratigraphy and fracture and void variation with depth, and compare these data with the change in P-wave velocity with depth.

A primary difficulty with interpreting lithostratigraphy of the oceanic crust from core samples recovered by the DV *Glomar Challenger* has been the low percentage of core re-

covery. Drilling operations crush and wash away an average of 70% of all oceanic crust drilled through. The only measurements capable of recording information over the entire wellbore are geophysical logs, but it has been extremely difficult to interpret these logs in detail because of a lack of comparable examples on land for cross-correlations (see ref. 6).

A borehole televiewer (BHTV) was used to provide the type of continuous data needed to interpret more fully the structure and stratigraphy of the ocean crust. The BHTV is a seldom-used oil-field logging tool developed by the Mobil Oil Corporation for the study of fractures in wellbores and casing (see ref. 7). The downhole tool contains an ultrasonic transducer which rotates three times per second and emits a 1 MHz pulse 180 times per second as the tool is moving vertically in the hole at a speed of 1.5 m min^{-1} . By displaying the amplitude of the acoustic pulse that is reflected off the borehole wall as brightness on a three-axis oscilloscope, the BHTV produces an image of the reflectivity and smoothness of the wellbore referenced to magnetic north. Hard, smooth surfaces produce strong reflections (or 'bright zones' on photographs of the data displayed on the oscilloscope) whereas fractures and voids greatly diminish the reflected signal and produce 'dark zones' on the records. Similarly, where the hole reaches great width, or very soft clays are encountered, no appreciable reflection is returned, and dark zones again appear. The result is a complete

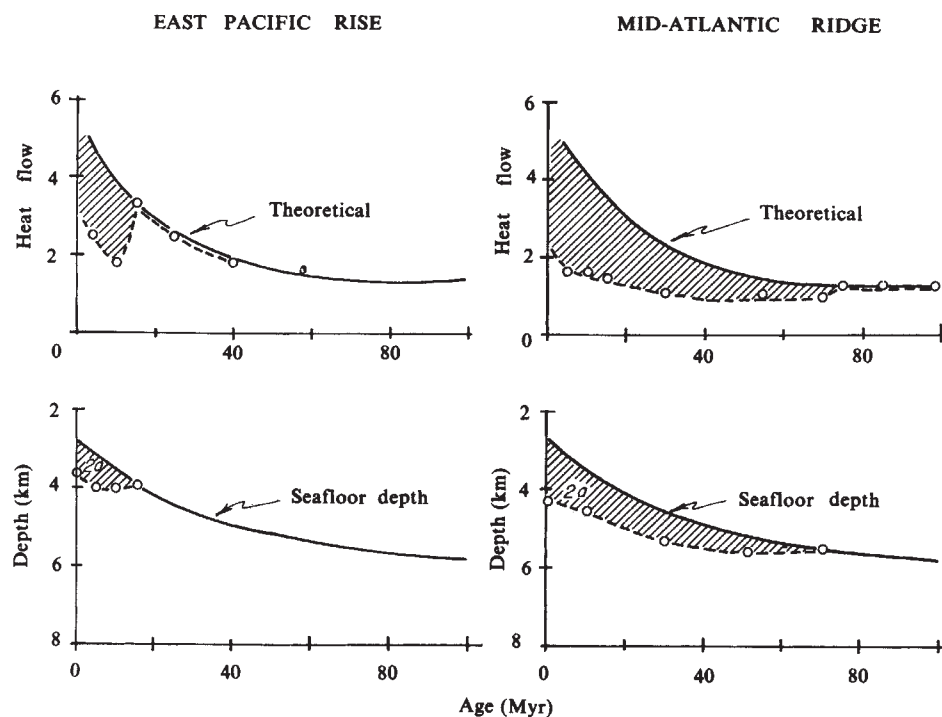


Fig. 1 Heat flow and thickness of seismic layer 2A as they vary with age in the Atlantic and eastern Pacific oceans^{1,2}. In the upper plots, $\circ$ represents mean observed heat flow for corresponding age; theoretical curve is prediction of lithospheric cooling model; hatching is heat believed to be carried away by convection through the sea floor. In the lower plots are layer 2A thickness, $\circ$ represents observations from sonobuoy data; hatching is thickness of layer 2A below sediment-rock interface.

north-east-south-west-north 'picture' of the reflectivity of the wellbore.

The setting

We deployed a borehole televiwer at sites 501 and 504B on IPOD Legs 68A and 69 in 6-Myr-old crust on the south flank of the Costa Rica Rift, the easternmost segment of the Galapagos Spreading Centre (Fig. 2). The sites were chosen to penetrate into young oceanic crust with high surface heat flow. The two sites are only about 1 km apart. Because they lie near the Equator where there is rapid productivity of pelagic sediment, a 280 m sedimentary section has formed a thick impermeable lid over a hydrothermal circulation system active in the oceanic crust³. Pore pressure measurements³ and temperature logs⁸ indicate oceanic bottom water being drawn naturally into an underpressured aquifer with a permeability of about 200 millidarcies (md) located 50–100 m below the top of layer 2A⁸. Flow tests in hole 504B show that the average permeability of layer 2A drops from 20–40 md in the upper 100 m to 2–4 md about 200 m below the sediment-basalt contact³. Sonic logs indicate an increase in velocity of layer 2A from 2.5 to $>5 \text{ km s}^{-1}$ in the upper 200 m of the oceanic crust.

The BHTV tests conducted in these wells have contributed to our understanding of why the sedimentary layer forms a hydraulic lid, why the permeability drops by an order of magnitude with depth and why the seismic velocity increases with depth. It also gave the first complete glimpse of the lithostratigraphy of the upper oceanic crust.

Structure at site 501

Using the BHTV we logged the entire sedimentary column and 25 m below the sediment-basalt contact in hole 501 of DSDP Leg 68A (Fig. 3). In much of the sedimentary section the record is quite dark (that is, there is no significant reflection from much of the circumferential sweep of the trace). This is due to pronounced 'washing out' and ellipticity of the hole; the problem is most severe at ~3,710 m depth (Fig. 3).

The youngest chert yet encountered by the *Glomar Challenger*⁹ was found over the lower 30 m of the sediment column of hole 501. The BHTV records show that the first chert encountered is a solid layer ~5 m thick. Drilling results indicated that within this layer there are interlayered lenses of chert and silicified limestone. The chert, being harder and more

resistant to 'washing out', shows up as bright, high-reflectance bands between the darker limestone layers. Although we do not know the horizontal extent of these chert layers, all holes drilled at the 501–504B sites encountered chert over the lower 30 m of the sedimentary section.

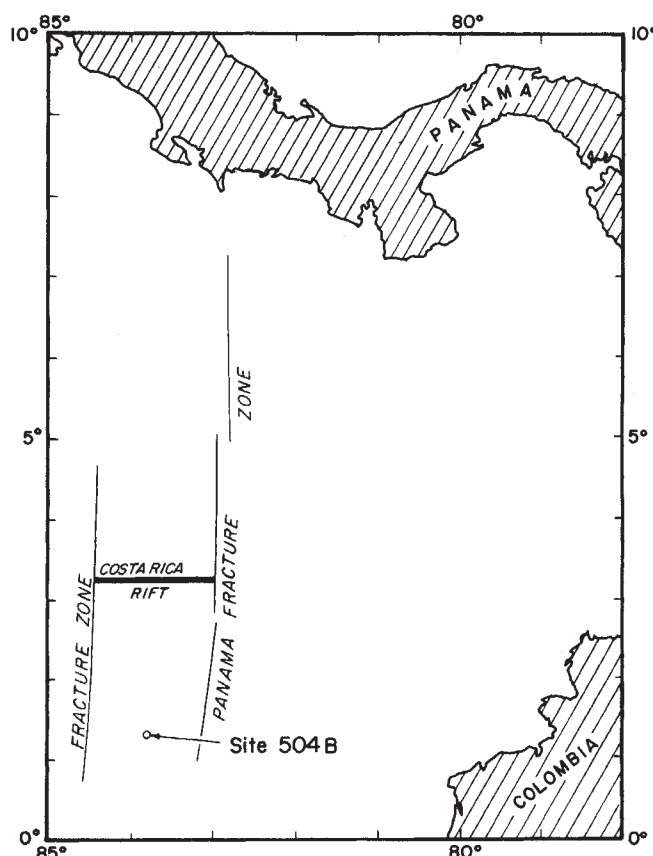


Fig. 2 Index map showing location of DSDP site 504B. 501 is 1 km to the west of 504B. The Costa Rica Rift is the easternmost segment of the Galapagos Spreading Centre.

Pillow basalts recorded by the BHTV appear as bright spots within dark ellipses. These dark ellipses probably represent the pillow's glass and alteration rims that are fractured and washed-out during drilling (Fig. 3). The pillow units as seen in the wellbore also seem to contain large voids where virtually no signal is reflected back to the transducer (for example, at 3,735 m in Fig. 3). Several massive flow units are seen at the top of the oceanic crust interlayered with the pillow basalt units. Because of the poor core recovery, the interrelationship between these stratigraphical units was not previously known. Note the 8 m thick massive flow unit that extends from 3,747 to 3,755 m.

Structure at site 504B

At site 504B on Leg 69, a BHTV was used to log 196 m below the casing which was set 2 m into basalt. Unfortunately, the factory-equipped transducer was destroyed on the first lowering of the tool by a pressure-compensation failure. The hole was then logged using a 600 kHz transducer array taken from an acoustic reentry tool as a replacement televiewer transducer. Sixty-cycle noise (apparently caused by a faulty logging cable) further obscured the BHTV image. Because the noise has proved difficult to remove from the data, we present only line drawings (Fig. 4) rather than the original records. Nevertheless, distinct pillow and flow units were discernible and contacts between distinct eruptive events were often seen (for example, 3,855 m, Fig. 4). Fracture resolution is several millimetres and several fractures this small were seen (for example, 3,860 m, Fig. 4).

The interpretative line drawing of the BHTV records allows us to reconstruct the lithostratigraphy of hole 504B (Fig. 4). We observe that thick flow units dominate the upper 50 m of layer 2A. These flows have numerous fractures which are predominantly horizontal to subhorizontal. Thin interlayers (~1 m thick) of large diameter pillow units are seen at 3,757, 3,765, 3,773–3,778 and 3,782 m. The 8 m thick massive flow unit of hole 501 (at 3,747–3,755 m of Fig. 3) may be correlative with a thick flow encountered at 504B at depths of 3,753–3,758 where it is more broken-up. Another thick massive flow is in 504B seen at 3,785–3,795 m depth.

The percentage of pillow units gradually increases down-section and the average size of the pillows decreases from 20 to 30 cm near the basalt-sediment contact to only a few centimetres (Fig. 4). For example, from 3,838 to 3,851 m a suite of pillow units has a range in sizes up to ~30 cm whereas the pillow units from 3,890 to 3,897 are all <10 cm in size. In addition, the massive flow units become thinner down-section. The entire lower 55 m of the recorded section appears as discrete pillow units <1 m in thickness. Fractures often seem to be concentrated at the borders of separate pillow flows but some zones are uniformly fractured. As in hole 501, most of the fractures observed in hole 504B are horizontal to subhorizontal.

From 3,805 to 3,825 m an anomalously low reflectance zone exists. While some structure can be seen within this zone, the amplitude of reflectance is much lower than zones directly above and below it. The general level of reflectance also drops from flows to pillows and towards the bottom of the hole. The lowered reflectance may be due to the increased occurrence of clay and zeolite which was clearly observed in the cores⁹.

Increase in seismic velocity with depth

A primary aim of this project is to interpret the BHTV records in terms of the change in the physical properties of the ocean crust. Specifically, we wish to relate variations observed in the sonic velocity log over the BHTV-logged section of 504B to the stratigraphic variations seen with the BHTV. Although the velocity log from hole 504B is extremely noisy, a marked increase in average P-wave velocity with depth can still be clearly seen (Fig. 5a, after ref. 4). The average basement velocity just below the casing begins at ~2.5 km⁻¹ and increases over the

next 200 m to ~5 km s⁻¹ (values typical of layer 2A). Although most of the large-amplitude high-frequency velocity variations in Fig. 5a are spurious and due to the effect that changes in the shape of the hole have on the seismic waveforms being transmitted by the velocity tool, the high-velocity interval from 3,785 to 3,795 m correlates with a massive flow unit which contains few fractures or voids at that depth.

Fracture and void counts

The BHTV record of hole 504B allows us to test various hypotheses for the increase in seismic velocity with depth within layer 2A because we can correlate the observed change in character and structure of fracture and void zones down the hole

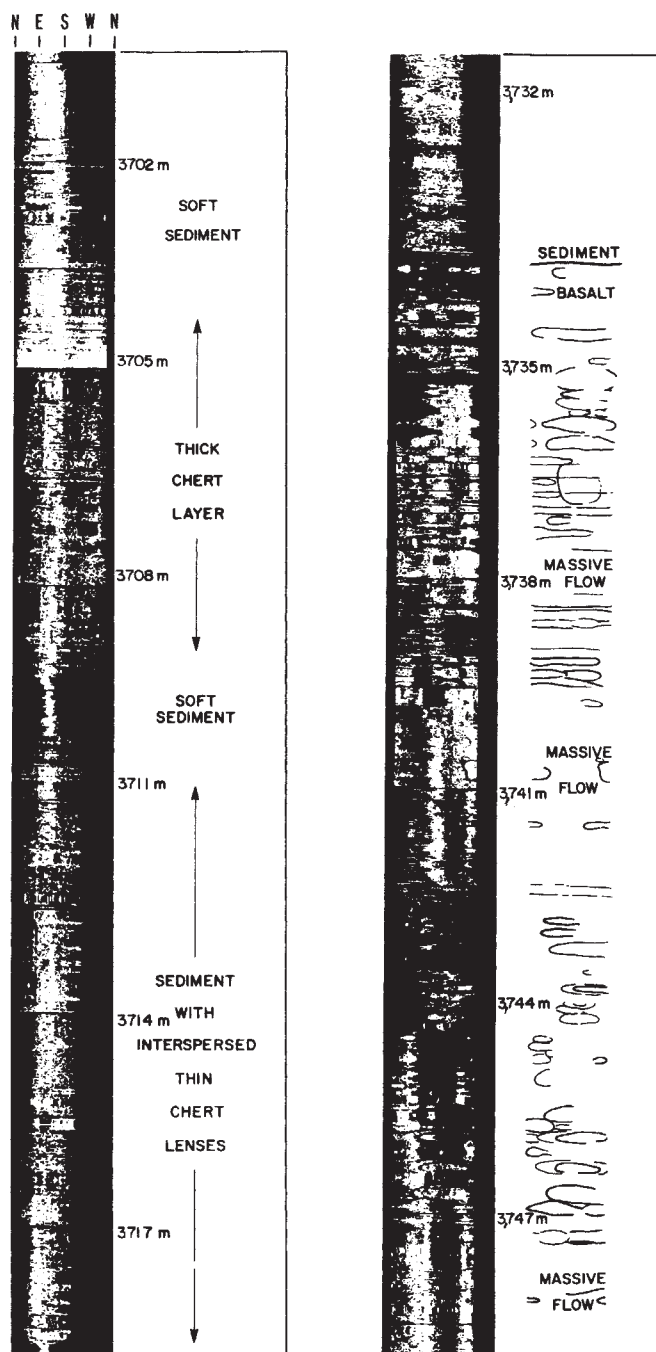


Fig. 3 Borehole televiewer imagery of the wellbore at site 501. Horizontal exaggeration is 1.5:1. The horizontal axis is north-east-south-west-north dissection of the hole. White is strong reflectance, black is weak reflectance. Note that the sedimentary section from 3,718 to 3,731 m is omitted.

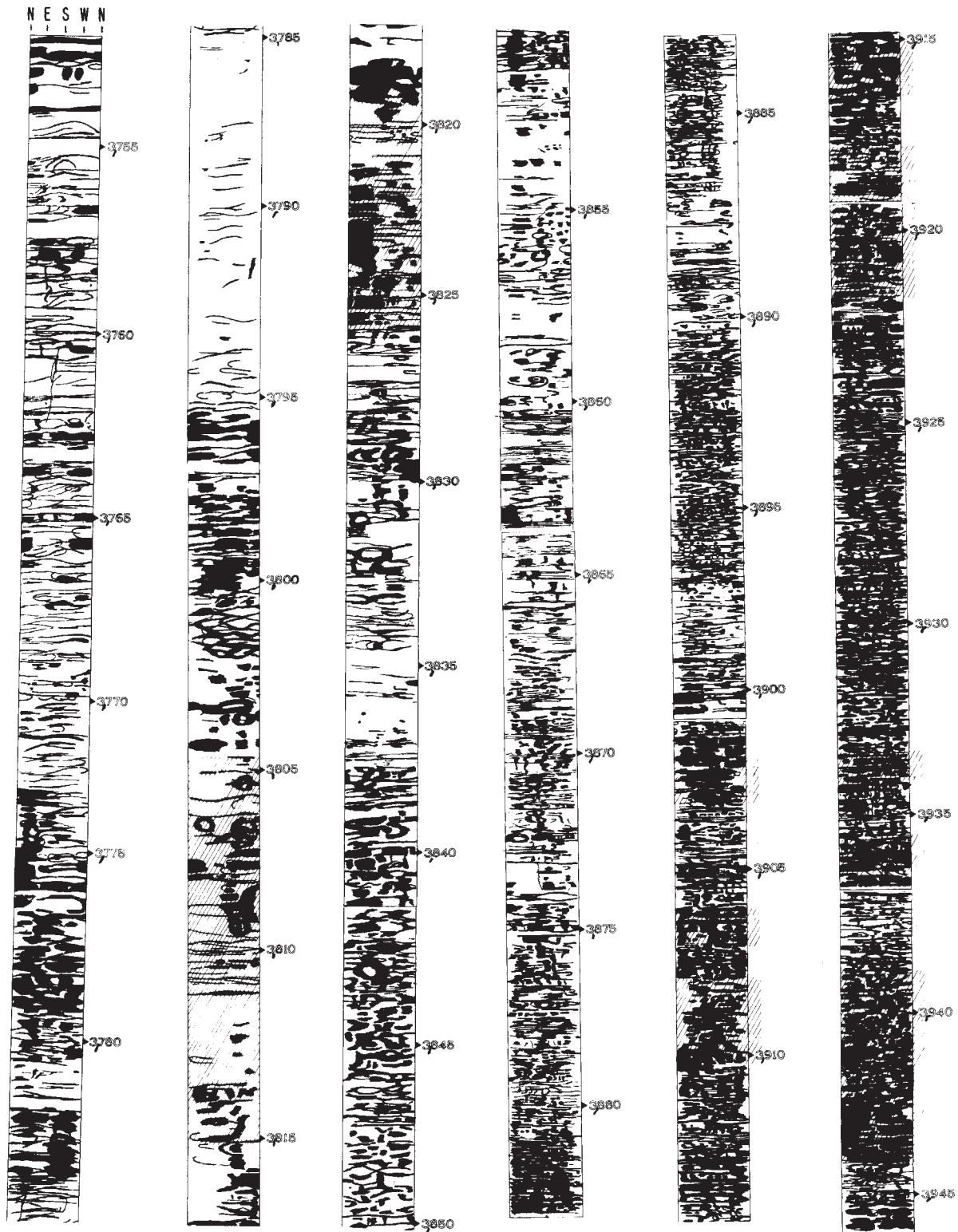


Fig. 4 Line drawing of borehole televiewer imagery of site 504B drillhole. Horizontal exaggeration is 1.5:1. Black-rimmed elliptical images are pillow lavas. Solid white segments are massive flow basalts. Thin lines are cracks and fractures. Hatched areas are of anomalously low reflectance. Depths in metres below sea level. Casing ends at 3,753 m.

with changes in seismic velocity. To this end, we counted the number of fractures observed on the BHTV records from hole 504B (Fig. 5b). As can be seen in both Figs 4 and 5b, the fracture density actually increases with depth in hole 504B. As laboratory studies show, this would be expected to decrease the velocity if the degree of fracturing alone were controlling V_p in layer 2A. Instead, a marked velocity increase with depth is observed in hole 504B that is typical of layer 2A (Fig. 5a).

As voids between pillows might be expected to have a similar effect on velocity as fractures, a plot of the percentage of voids in the wellbore (percentage of black versus white in Fig. 4) is presented in Fig. 5c. In the pillow units, this 'void ratio' (Fig. 5c) measured the per cent of pillow rims, glassy rind and rubble surrounding the solid basaltic core of each pillow. We observe an increase of voids with depth in the hole because of the decrease in the diameter of the individual pillows with depth. Presumably,

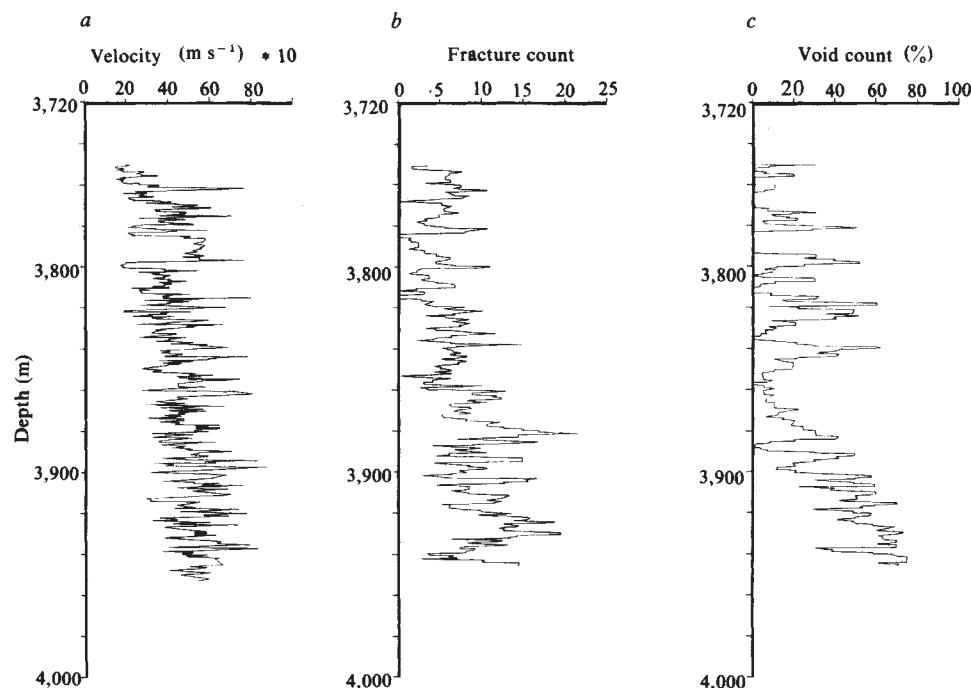


Fig. 5 *a*, Sonic velocity logs of site 504B upper 200 m of basalt corresponding to interval of BHTV log in Fig. 4. Spurious data points with $V_p < 1.5$ and $> 8 \text{ km s}^{-1}$ were excluded from digitization of original analog record. Note that despite the noisy character of the log a clear increase in V_p with depth can be observed in hole. *b*, Histogram of the number of fractures per 50 cm of hole. *c*, Void percentage based on an estimation of the per cent black versus white in Fig. 4 per 50 cm of hole.

a lower seismic velocity might be expected to correlate with the higher void count, but again, an inverse correlation is seen in hole 504B.

Discussion

To explain the variation of velocity in the upper oceanic crust three basic questions must be answered. Why is the velocity of the upper portion of layer 2A so low? Why does the velocity increase so rapidly with depth within layer 2A? And why does layer 2A disappear with age at about the time of the transition in the mode of heat transfer along ridge flanks? It has been argued¹⁰⁻¹³ that the low seismic velocity of the upper portion of layer 2A results from the highly fractured and porous nature of the shallow oceanic crust. This explanation seems quite reasonable from the nature of the basalt encountered in holes 501 and 504B. However, what causes the velocity to increase so rapidly with depth? Velocity is expected to increase with depth due to the closure of fractures with pressure, but the increase in effective pressure over only a few hundred metres depth is quite small (only about 15–20 bar per 100 m), and mechanically closing enough fractures and voids to account for the velocity increase over the depth range of interest is highly unlikely¹². Spudich and Orcutt¹⁴ suggested that the increase in velocity is caused by a decrease in porosity with depth. But why should a decrease in porosity occur in a regular manner on the flanks of many ridges, and how could this account for the disappearance of layer 2A with age?

Schreiber and Fox¹² have proposed a model that seems to explain the seemingly inconsistent seismic velocity and borehole televiwer data in holes 501 and 504B, as well as several of the questions posed above. They suggested that as voids and fractures are filled by alteration minerals like chlorite, zeolite and smectite (with characteristic seismic velocities of $\sim 4 \text{ km s}^{-1}$) the alteration minerals replace seawater (with a seismic velocity of

$\sim 1.5 \text{ km s}^{-1}$). Thus, the seismic velocity of layer 2A would be expected to rise rapidly as the degree of alteration increased. Increased alteration in layer 2A would be expected with both depth (due to increased temperature) and age. Geophysical logging data⁶ also suggests that an increase in alteration-filling occurs with age on ridge flanks. The alteration-filling hypothesis seems capable of explaining the rapid increase of layer 2A velocity with depth despite an apparent increase in fractures and voids and the eventual disappearance of layer 2A with age. The increased occurrence of clays and zeolites with depth was well noted in the core descriptions of holes 501 and 504B⁹ and the rapid decrease in permeability with depth in hole 504B³ also indicates the filling of fractures and voids with alteration minerals. We do not now understand the cause of the decrease in the size of the pillows encountered in 504B and the related increase in the number of voids. This may be a localized phenomena which is of no general significance. However, the observation of a marked increase in velocity despite an apparent increase in fractures and voids demonstrates that the replacement of seawater by alteration minerals is more important in controlling the increase in velocity than the change in fracture or void density. If this is correct, it suggests that on the flanks of oceanic spreading centres a marked decrease in the permeability of the 'crustal plumbing system' can be expected to correspond to the change in seismic velocity. This would explain the correlation between the transition from conductive to convective heat flow and the disappearance of layer 2A with age.

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Resolving the functions of overlapping viral genes by site-specific mutagenesis at a mRNA splice site

Craig Montell*, Eric F. Fisher[†], Marvin H. Caruthers[†] & Arnold J. Berk*

* Department of Microbiology and Molecular Biology Institute, University of California, Los Angeles, California 90024, USA

[†] Department of Chemistry, Campus Box 215, University of Colorado, Boulder, Colorado 80309, USA

Early region 1A of human adenoviruses encodes a function required for normal induction of early viral genes and virus-induced cell transformation. The region is expressed at early times as two overlapping spliced mRNAs, 12S and 13S, which encode closely related proteins. To distinguish between the functions of these proteins, a single T → G transversion was constructed which prevents splicing of the 12S mRNA. This transversion, in the second base of the 12S mRNA intron, does not alter the protein encoded by the 13S mRNA due to degeneracy in the genetic code. Studies with this mutant demonstrated that only the 13S mRNA encodes the regulatory protein required for normal early gene expression.

MANY eukaryotic genes are interrupted by introns removed from mRNA precursors by RNA splicing. Sequences encompassing splice sites share considerable homology and variants of the following sequence are found at the 5' end of every intron analysed: 5'-(exon)-AG/GUAAGUA-(intron)-3' (refs 1-3). Although specific 5' splice sites differ from this consensus sequence to a greater or lesser degree, the underlined GU dinucleotide which begins the intron is virtually invariant. To determine whether the integrity of this dinucleotide is required for efficient RNA splicing, we constructed an adenovirus mutant with a T → G transversion in the second base of the intron in an early region 1A (E1A) gene. We believed that this transversion would eliminate RNA splicing, allowing us to ascribe specific functions to the two overlapping early adenovirus mRNAs transcribed from this region⁴. Animal viruses frequently encode overlapping spliced mRNAs in a common region of the viral genome. Such compact gene organization enables the small viral genomes to encode several related proteins in the same region by splicing a single mRNA precursor in two or more alternative ways.

E1A of adenovirus 2 (Ad2) has attracted considerable interest because it encodes a function required for both normal expression of the other early viral genes^{5,6} and virus-induced cell transformation^{7,8}. The two overlapping early mRNAs transcribed from this region, 12S and 13S, share identical 5' and 3' termini and vary by 138 nucleotides due to the differing sizes of the introns removed by RNA splicing (refs 9, 10; Fig. 1). Assuming translation is initiated at the first AUG, the 12S and 13S messages encode proteins of 243 and 289 amino acids respectively. The 12S and 13S mRNAs are spliced such that they continue in the same reading frame⁹; therefore, the larger protein has the same amino acid sequence as the smaller protein except for an additional internal 46 amino acids encoded by the 138 nucleotides unique to the 13S mRNA. One E1A mutant, Ad5 hr1 (ref. 11), has a single base deletion in the portion of viral genome encoding the unique region of the 13S mRNA¹². This mutation leads to premature chain termination during translation of the 13S mRNA, but does not affect translation of the 12S mRNA¹². Ad5 hr1 is deficient in both early viral gene expression⁵ and inducing cell transformation⁷, demonstrating that the 289 amino acid protein at least is required for these processes.

The question of the role of the 243 amino acid protein in regulating early viral gene expression or virus-induced cell transformation has not previously been addressed. It cannot be investigated using mutants of the 243 amino acid protein as random or directed mutagenesis intended specifically to alter

the 243 amino acid protein encoded by the 12S mRNA, without affecting the 289 amino acid protein specified by the 13S mRNA, would be futile since every amino acid in the smaller polypeptide is included in the larger one. To overcome this

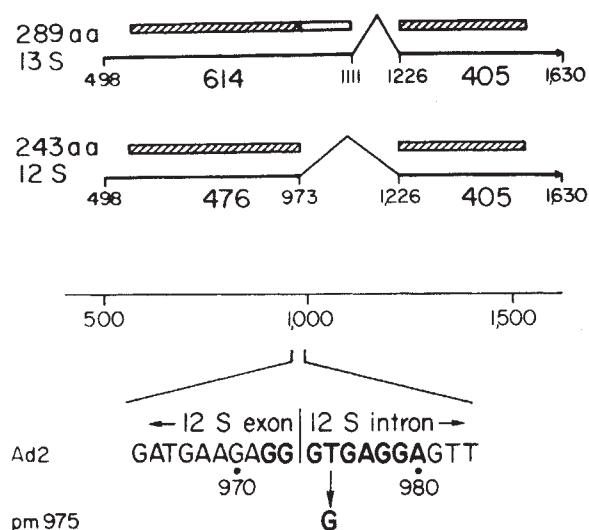
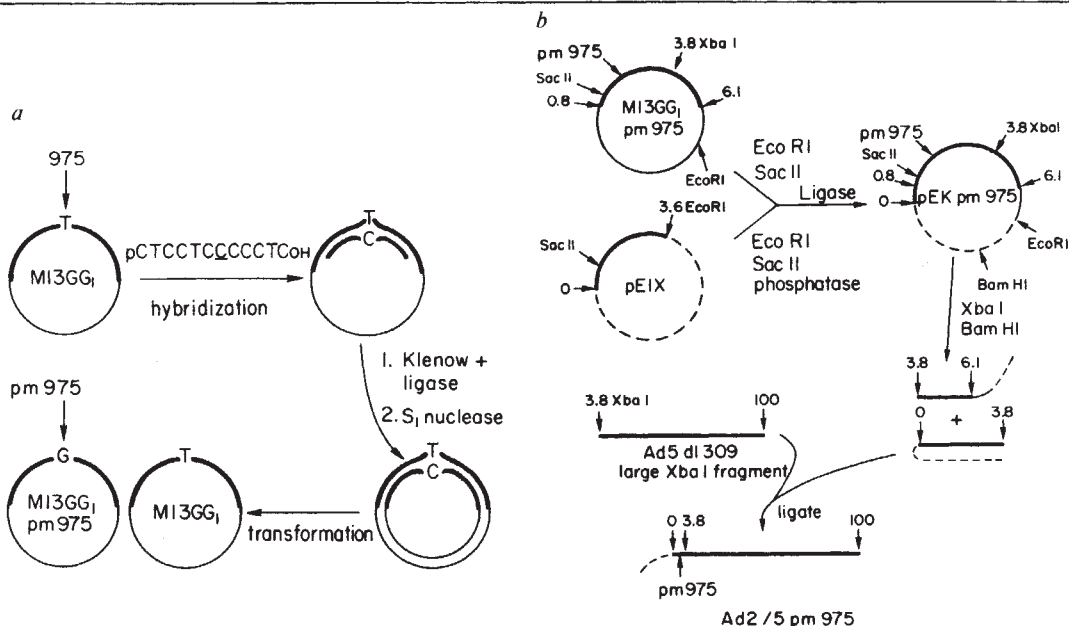


Fig. 1 Structures of E1A gene products at early times. The E1A portion of the Ad2 genome is represented as a line at the centre of the figure marked off in base pairs from the left end of the genome. The horizontal lines joined by carot symbols represent exons of the 12S and 13S mRNAs; the total lengths of exons are indicated in nucleotides below the lines with the end points identified in the Ad2 sequence; arrowheads indicate the 3' ends of the mRNAs. Boxes above the exons demarcate the translated regions. The lengths of the polypeptides are given as the number of amino acids (aa). The portions of the 289 and 243 amino acid proteins with identical sequences are indicated with hatches; the open box refers to the 46 amino acids unique to the larger polypeptide. The sequence of the L strand of Ad2 in the vicinity of the 12S mRNA 5' splice site between nucleotides 964 and 983 is shown below the line representing the genome. Those nucleotides included in the 5' consensus sequence are highlighted. The vertical line identifies the splice junction separating the 5' 12S exon from the intron and the T → G transversion at nucleotide 975 is indicated. The Ad2 E1A sequence is >99% homologous with the corresponding region in Ad5 (ref. 36). To align the Ad2 with the Ad5 E1A sequence, add one to the Ad2 sequence to account for an additional base pair in the Ad5 inverted terminal repeat.

Fig. 2 Construction of Ad2/5 pm975. **a**, Oligonucleotide-directed mutagenesis. The bold portion of the circles represents the Ad2 component. T refers to nucleotide 975 in the Ad2 sequence and the underlined C to the corresponding mismatch in the dodecanucleotide. M13GG1 was constructed (by R. Gaynor) by transferring the Ad2 *KpnI* fragment from pSB2 (ref. 37) into the *KpnI* site of M13Gor1 (ref. 38) producing a clone of the Ad2 L strand extending from nucleotides 269 to 2,049. Oligonucleotide-directed mutagenesis was performed as described elsewhere¹⁵ with M13GG1 as the single-stranded template and *E. coli* K12 JM101 (ref. 39) as host, except for the following modification. After 4 h of DNA synthesis, the specified amounts¹⁵ of DNA polymerase I large fragment



(Klenow) and T4 DNA ligase were added again and incubation continued for 3 h. Transfection was into CaCl_2 treated cells³⁸ rather than spheroplasts. We found that single-stranded viral DNA had an infectivity of $\sim 10^3$ plaque-forming units (PFU) per μg compared with $\sim 10^6$ PFU per μg for double-stranded DNA in CaCl_2 -treated JM101. The dodecanucleotide primer was synthesized as described in ref. 16 with the following modifications. The support was prepared by coupling 5'-O-(dimethoxytrityl)-3'-(4-p-nitrophenyl succinyl)-N-benzoyldeoxycytosine to silica treated with 3'-(triethoxysilyl)propylamine. The activated nucleoside was prepared at room temperature and used immediately. Synthesis was carried out in a modified Merrifield apparatus using 150 mg of derivatized support ($46 \mu\text{mol g}^{-1}$). At each condensation $90 \mu\text{mol}$ of activated nucleoside was used. Fifty mg of the resulting silica covalently attached to the deoxyoligonucleotide was treated for 90 min with thiophenol/triethylamine/dioxane (1:1:2). The silica was isolated by centrifugation and washed four times with dioxane, three times with methanol and once with diethylether. $400 \mu\text{l}$ of concentrated ammonium hydroxide were added to the silica. The sealed tube was shaken for 3 h at room temperature followed by centrifugation. The supernatant was transferred to another tube, sealed and heated to 50°C for 22 h. The sample was dried *in vacuo* and $250 \mu\text{l}$ of 80% acetic acid were added. After 60 min, the sample was dried *in vacuo* and applied to a $1.5 \text{ cm} \times 50 \text{ cm}$ G50/40 column in 0.01 M triethylammonium bicarbonate. Fractions (1.2 ml) were taken and the first three fractions containing UV-absorbing material ($\sim 10A_{260}$) were pooled, lyophilized to dryness, dissolved in $150 \mu\text{l}$ of 80% formamide and applied to a $20 \text{ cm} \times 20 \text{ cm}$ 20% polyacrylamide/7 M urea gel. After electrophoresis the band containing the desired oligodeoxynucleotide was excised, eluted and desalted on a Bio-Gel P 2 column ($3 \text{ cm} \times 60 \text{ cm}$). The 2.5-ml fractions were monitored by UV absorption. The fractions containing the compound were pooled (4.4 A_{260}). The sequence of the oligonucleotides was verified by two-dimensional TLC as described previously⁴⁰ using Homomix V. **b**, Insertion of pm975 into the complete adenovirus genome. Bold lines represent adenovirus sequences, light lines M13Gor1 DNA and broken lines pBR322 sequences. Small numbers refer to Ad2 map units; one map unit is equal to 1% of the Ad2 genome— ~ 350 nucleotides. A derivative of pBE5, pEIX¹⁹, which contains Ad2 sequence from 0 to 3.6 map units inserted between the *PstI* and *EcoRI* sites of pBR322 (ref. 41), was prepared. The mutation in M13GG1 pm975 was introduced into pEIX by digesting $0.5 \mu\text{g}$ pEIX with *SacII*, *EcoRI* and calf intestinal phosphatase; ligating the products of these reactions to $5 \mu\text{g}$ *SacII*, *EcoRI*-digested double-stranded M13GG1 pm975; and using the reaction products to transform *E. coli* C600 to Tc^r all as described in ref. 37. Plasmids from transformed colonies were screened by size and restriction endonuclease analysis to identify the desired construct, pEK pm975, which includes the entire left terminus of Ad2. The presence in this plasmid of the T $\rightarrow$ G transversion at 975 in the Ad2 sequence was confirmed by DNA sequencing^{17,18}. $4.5 \mu\text{g}$ pEK pm975 were digested with *XbaI* and *BamHI* (to minimize recircularization) and ligated to $1 \mu\text{g}$ of the large *XbaI* fragment of Ad5 d1309 isolated as described in ref. 19. Ad5 d1309 is a phenotypically wild-type variant of Ad5 containing a single *XbaI* site at 3.8 map units. The ligation creates complete 35 kilobase infectious adenovirus genomes, which include the T $\rightarrow$ G transversion at base pair 975. The ligation products were transfected into 293 cells by the calcium phosphate technique⁴². Ad2/5 pm975 was collected from a well isolated plaque and the plaque was purified a second time on 293 cells.

problem we used oligonucleotide mutagenesis to create a T $\rightarrow$ G transversion at position 975 in the Ad2 sequence (Fig. 1). This nucleotide, which is the second base in the 12S mRNA intron, is also the third base of the GGU codon specifying glycine in the overlapping 13S mRNA. Because there is degeneracy in the genetic code, the new codon, GGG, created by this mutation also specifies glycine, thereby leaving the 289 amino acid protein completely unaltered.

We find that the single base change prevents efficient RNA splicing and specifically eliminates the 12S mRNA. Studies with this mutant, Ad2/5 pm975 (pm = point mutation), demonstrate that the 13S mRNA encodes all the early EIA functions required for normal early gene regulation and virus replication in human cells.

Construction of the specific adenovirus point mutant

The T $\rightarrow$ G transversion was introduced into a bacteriophage M13 clone of EIA by oligonucleotide-directed mutagenesis^{13,14}. Conditions for this site-specific mutagenesis have been optimized by Gillam *et al.*¹⁵ for isolating a mutant with a single point mutation at high efficiency without biological selection. The method (Fig. 2a) entails hybridization of a synthetic oligonucleotide to a single-stranded circular bacteriophage genome. The oligonucleotide is complementary to the phage DNA except at a single predetermined nucleotide. The hybridized oligonucleotide is used as a primer for DNA polymerase, and the resulting duplex molecules are ligated to form infectious covalently closed circles. In this work, the mutating oligonucleotide was a dodecanucleotide (12 mer) synthesized on a solid support by a modification of the phosphite coupling method of Mattucci and Caruthers¹⁶. The 12 mer is complementary to the L strand of EIA encompassing the 12S mRNA 5' splice site except for a mismatch at nucleotide 975 in the Ad2 sequence. The heteroduplexes were transfected into *Escherichia coli* giving rise to a mixed population of mutant and wild-type

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Table 1 Host range analysis of pm975

Virus	293	PFU per ml	
		HeLa	HeLa/293
Wild-type Ad2	6.63×10^9	8.71×10^9	1.3
Ad2/5 pm975	1.38×10^9	1.76×10^9	1.3
Virus	HEK	HEK/HeLa	
		HeLa	HEK/HeLa
Wild-type Ad2	1.17×10^{10}	1.46×10^{10}	0.80
Ad2/5 pm975	1.96×10^9	3.03×10^9	0.65

Stocks of Ad2 and plaque-purified pm975 prepared on 293 cells were serially diluted and assayed in duplicate by plaque formation on 293 and HeLa cells, and HEK and HeLa cells. Titres are based on the average of duplicate plates yielding 30–300 plaques.

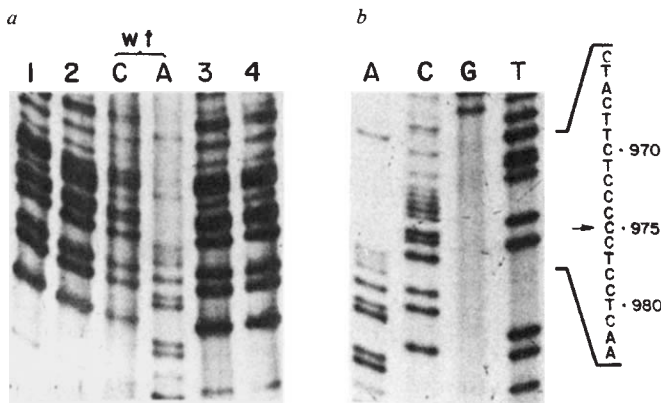


Fig. 3 Identification of M13GG1 pm975 and Ad2/5 pm975 by dideoxy sequencing. *a*, Following the second cycle of enrichment¹⁵, bacteriophage were collected from eight plaques and amplified overnight in 25-ml cultures of JM101; phage were precipitated with polyethylene glycol³⁶, and viral DNA was isolated as described previously⁴³. Purified DNAs were screened for the mutation by reverse transcriptase dideoxy sequencing using dideoxy CTP. The primer was a 31-base *Hpa*II fragment extending from nucleotides 1,009 to 1,040 in the Ad2 sequence. The sequences of four of the eight phage DNAs screened are shown in tracks 1, 2, 3 and 4 adjacent to the wild-type C and A lanes. Sequences shown are of the R strand of Ad2 in the vicinity of the 12S mRNA 5' splice site. The DNA in track 2 contained the desired mutation at nucleotide 975. *b*, Identification of Ad2/5 pm975. Adenovirus DNA was prepared as described elsewhere³⁷ from 293 cells infected with virus from well isolated plaques picked from a transacted 293 plate (see Fig. 2 legend). The DNA was digested with exonuclease III creating single-stranded termini ~1,500 nucleotides long⁴⁴. Sequence analysis was by the dideoxy method using the same primer as for *a*. The 20 nucleotide sequence in the vicinity of the 12S mRNA 5' splice site is listed and the positions of nucleotides 970, 975 and 980 are indicated.

clones. The heterogeneous population of DNA molecules was subjected to two cycles of enrichment by performing the oligonucleotide hybridization in conditions favouring hybridization to the mutant sequence¹⁵. Potential mutant clones were screened by DNA sequencing by the dideoxy chain termination method using dideoxy CTP^{17,18}. Of eight phage plaques thus screened, seven were wild type and one (Fig. 3*a*, lane 2) had the desired point mutation at base pair 975 (M13GG1 pm975). The mutation was introduced into a phenotypically wild-type adenovirus genome, Ad5 d1309, by the method of Stow (ref. 19; Fig. 2*b*).

Unable to predict whether the mutation at base pair 975 would eliminate an essential function, we transfected the reconstructed mutant genome into 293 cells which are capable of complementing all essential EIA functions^{8,20}. 293 cells are a line of Ad5-transformed human embryonic kidney cells containing an integrated copy of the left 12% of the Ad5 genome²¹ which constitutively express EIA and EIB mRNAs⁵ and proteins²². DNA sequence analysis showed that three out of four plaques screened contained the point mutation (Fig. 3*b*). The mutant, Ad2/5 pm975, was plaque-purified a second time on 293 cells and sequenced again to confirm the desired base pair change.

12S mRNA is eliminated

EIA mRNAs were assayed by the hybridization/*S*₁ nuclease technique²³. Early cytoplasmic RNAs were isolated 6 h post infection from HeLa cells infected with Ad2 or Ad2/5 pm975 and hybridized in DNA excess to a ³²P-labelled single-stranded M13 clone of the coding strand of EIA and the 5' 350 nucleotides of EIB. The hybridization products were digested with *S*₁ nuclease and the protected fragments were resolved by electrophoresis on a denaturing polyacrylamide gel and detected by autoradiography.

Figure 4*a* displays the DNA fragments protected by hybridization to the wild-type EIA and EIB mRNAs. The 614 nucleotide fragment corresponds to the 5' exon of the 13S mRNA, the 476 base band to the 5' exon of the 12S mRNA, the 405 nucleotide fragment to the 3' exon common to both the 12S and 13S mRNAs and the 350 nucleotide protected fragment to

the terminal portion of the 5' exons of EIB mRNAs. An additional but minor band of 750 nucleotides is protected by RNA species identical to the 13S mRNA except for a ~140 nucleotide extension at the 5' end²⁴.

*S*₁ analysis with RNA prepared from pm975-infected cells reveals bands co-migrating with each of the wild-type Ad2 bands except for the 476 nucleotide protected fragment corresponding to the 5' exon of the 12S mRNA (Fig. 4*a*). To enhance the sensitivity for detecting the 12S mRNA, poly(A)⁺ RNA isolated from cells during the late stage of infection was analysed (Fig. 4*b*). The level of 12S and 13S mRNA is increased approximately twofold during the late phase in cells infected with wild-type virus. In addition, a new 9S spliced mRNA having the same 3' exon as the 12S and 13S mRNAs and a still shorter 5' exon becomes the predominant form of EIA message^{25,26}. This gives rise to the increase in the proportion of protected DNA in the 405 nucleotide fragment and may account for the *S*₁ protected fragment of ~250 nucleotides. The level of EIB mRNAs is also increased several fold during the late phase²⁵. Despite these changes in the pattern of *S*₁-resistant

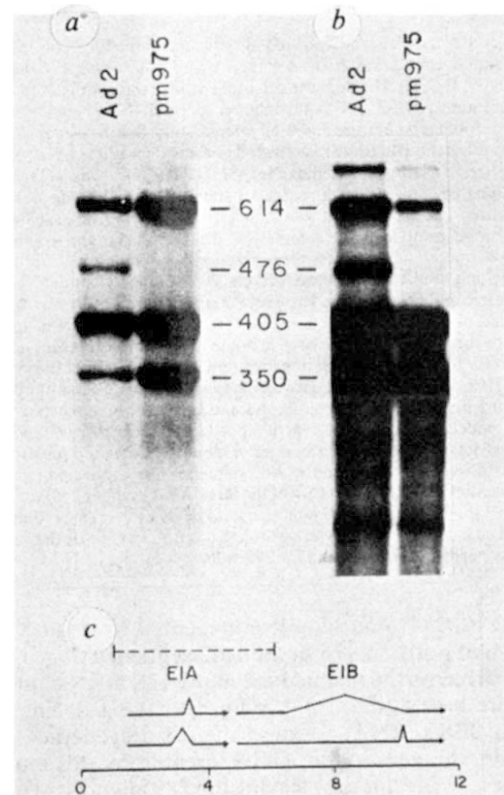


Fig. 4 Hybridization/*S*₁ nuclease analyses of EIA and EIB mRNAs from pm975 and Ad2. *a*, EIA and EIB early mRNAs. Cytoplasmic RNA was collected as described previously⁵ from 500-ml suspension cultures of log phase HeLa cells 6 h after infection with pm975 or Ad2 at 20 PFU per cell. 350 µg cytoplasmic RNA was hybridized to 0.028 µg M13-EIA single-stranded DNA labelled with ³²P to a specific activity of 3 × 10⁶ d.p.m. µg⁻¹ (ref. 45) for 30 min at 68 °C in 50 µl 1.0 M NaCl, 10 mM Tris pH 7.4, 1 mM EDTA. *S*₁ nuclease digestions in 500 µl were as described previously²³ except that digestions were with 15 Vogt units⁴⁶ per reaction at 37 °C for 30 min. Protected fragments were denatured, fractionated by electrophoresis on 14-cm 8 M urea-5% polyacrylamide gels for 15 h at 60 V, dried and visualized by autoradiography with an intensifying screen. The sizes of the predominant bands are indicated in nucleotides. *b*, Analysis of late poly(A)⁺ EIA and EIB cytoplasmic RNAs. Poly(A)⁺ cytoplasmic RNA (20 µg) isolated from late infected cells⁴⁷ was analysed as described in *a*. The autoradiogram was exposed for 17 days with an intensifying screen. *c*, M13 EIA single-stranded DNA probe (R. B. Gaynor and A.J.B., in preparation). The M13 EIA probe is similar to M13GG1 except that the R strand of Ad2 is cloned into the viral strand of M13Gor1. The long solid line represents a portion of the adenovirus genome marked off in map units. The horizontal lines joined by carot symbols refer to the exons of the major mRNAs transcribed from EIA and EIB at early times. Arrowheads indicate the 3' ends of mRNAs. The broken line delineates the adenovirus sequence included in the M13 EIA single-stranded probe.

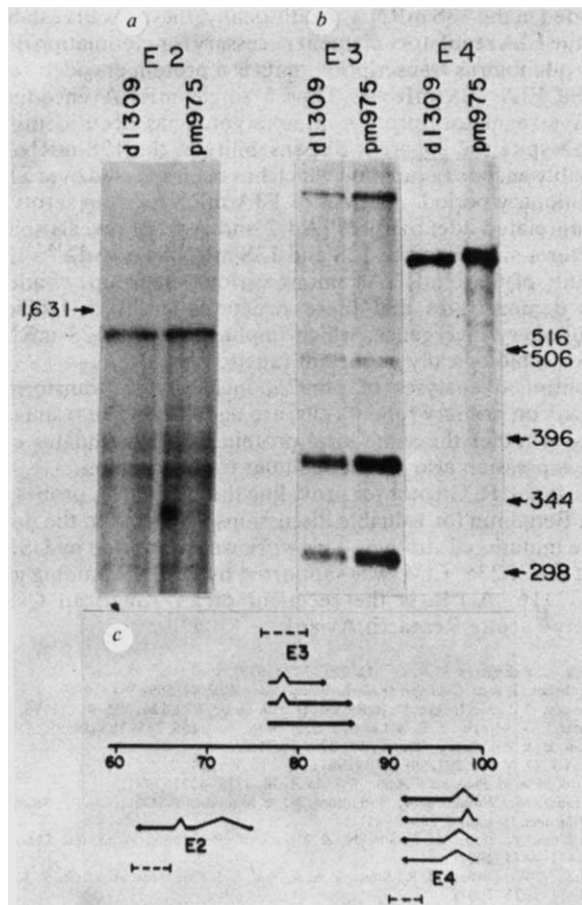


Fig. 5 Hybridization/ S_1 nuclease analysis of mRNAs from early regions 2, 3 and 4. Five μg of early poly(A)⁺ cytoplasmic RNA, prepared from HeLa cells 6 h post-infection with pm975 or d1309, were hybridized to M13 E2, E3 or E4 probes equivalent to 0.2 μg of the complete adenovirus genome. S_1 digestion and gel electrophoresis were as described for Fig. 4a. **a**, Early region 2 mRNA. S_1 -protected fragments were electrophoresed for 18 h at 120 V. The position of the 1,631 nucleotide *Hinf*I pBR322 marker is indicated. **b**, Early region 3 and 4 mRNAs. The protected fragments were fractionated by electrophoresis for 18 h at 50 V. Although the patterns of protected fragments are identical for d1309 and pm975, the structures of the E3 mRNAs differ from wild-type Ad5 due to a substitution and deletion in the E3 DNA sequence of d1309 (ref. 8). The positions of *Hinf*I pBR322 fragments are included to the right. **c**, M13 E2, E3 and E4 single-stranded probes (R. B. Gaynor and A.J.B., in preparation). The M13 E2 probe was constructed by inserting a *Kpn*I-*Xho*I fragment of Ad2 DNA (61.3–66.0 map units) between the *Kpn*I and *Xho*I sites of M13Gor1. The E3 probe was prepared by cloning an *Eco*RI-*Kpn*I fragment of Ad5 DNA (75.9–81.0 map units) between the *Eco*RI and *Kpn*I sites of M13Gor1. To construct the E4 probe an *Eco*RI-*Bal*I fragment of Ad2 DNA (89.7–93.2 map units) was cloned between the *Eco*RI and *Pvu*II sites of M13Gor1. The Ad5 genome and major E2, E3, and E4 early mRNAs are diagrammed as in Fig. 4c. Abundant mRNAs are represented by the bold lines with minor mRNA species indicated by light lines. Broken lines identify the adenovirus sequence included in each of the probes.

fragments at late times, no evidence of the 476 nucleotide S_1 protected fragment is observed in the experiment with pm975 (Fig. 4b). Thus, within the sensitivity of the S_1 analysis, the 12S mRNA is eliminated. Based on quantitative densitometry of these autoradiograms, we estimate that it would be possible to detect the 12S mRNA in pm975-infected cells if it were above 2% the level observed in infected wild-type cells.

These results (Fig. 4) prove that substitution of a G for U in the second base of the EIA 12S mRNA intron inhibits RNA splicing at least 50-fold. As the 13S EIA mRNA is produced in pm975-infected cells, the apparent elimination of the 12S mRNA must have been due to interference with RNA splicing. Interference with any other step in RNA processing would have eliminated the 13S mRNA as well, because it is processed from the same mRNA precursor.

13S mRNA encodes all pre-early functions required for normal growth on HeLa cells

Characterization of the functions encoded in EIA and EIB has been facilitated by adenovirus host range mutants selected for their diminished capacity to grow on HeLa cells relative to 293 cells¹¹. These mutants fall into two complementation groups (I and II) which map to EIA and EIB respectively²⁷. Host range mutants were used to show that EIA encodes a regulatory element^{5,6} and that functions encoded in EIA and EIB are required for viral transformation on primary rodent cells^{7,8}. One of the motivations for creating pm975 was to define whether one or both of the overlapping EIA mRNAs encode the functions required for virus replication in HeLa cells.

Plaque assays of pm975 and a wild-type control on HeLa and 293 cells (Table 1) establish that pm975 is not host range, and therefore the 12S mRNA is not required for plaque formation on HeLa cells. These data, together with studies of Ad5 hr1 discussed previously, show that only the 289 amino acid, and not the 243 amino acid, polypeptide is required for efficient replication of adenovirus 2 or 5 in HeLa cells.

Because HeLa cells are a continuous cell line, it was considered possible that a requirement for the 243 amino acid protein was masked by the transformed phenotype of these cells. Therefore, plaque assays were repeated with pm975 on HEK cells, a secondary cell culture of human origin, and compared with titres on HeLa cells. The results (Table 1) show that the 12S mRNA is not required for virus replication on HEK cells either.

13S mRNA encodes all pre-early viral regulatory proteins

The pattern and levels of mRNA induced by pm975 and a phenotypically wild-type control (either d1309 or Ad2) were compared using the hybridization/ S_1 nuclease technique. The hybridizations were performed between M13Gor1 single-stranded DNAs containing portions of the coding strand of each of the early transcription units and early cytoplasmic RNAs extracted from infected HeLa cells. Analysis of the fragment bands protected from S_1 digestion reveals no quantitative or qualitative differences between the early mRNAs induced by pm975 and Ad2 or Ad5 d1309 (see Fig. 4a for EIB, Fig. 5 for E2, E3 and E4). These results prove that the EIA regulatory elements that modulate early transcription are not encoded by the 12S mRNA and therefore must be specified by the 13S mRNA only.

Conclusions

A single U $\rightarrow$ G transversion introduced into the ubiquitous G-U at the 5' splice site succeeded in eliminating the 12S mRNA in EIA, leaving the overlapping 13S mRNA intact. This demonstrates that a single nucleotide alteration in the 5' consensus sequence is sufficient to interfere with the RNA splicing apparatus.

The fastidious nature of the splicing apparatus for an unaltered 5' consensus sequence has also been demonstrated in studies with a nitrous acid-derived Ad5 mutant, Ad5 hr440, which similarly fails to express the 12S EIA mRNA²⁸. This mutant has two alterations in the 5' consensus sequence, at positions five and six of the 12S intron (nucleotides 978 and 9'9 in the Ad2 sequence). One of these base pair changes creates a termination codon in the 13S mRNA, disrupting translation of the EIA 289 amino acid protein. Therefore, Ad5 hr440 is not useful in discriminating between the functions encoded in the two overlapping early EIA mRNAs.

Analysis of naturally occurring mutations in the β -globin gene from patients with β -thalassaemia has provided evidence that the integrity of the consensus sequence is also required for efficient splicing of cellular mRNAs. Restriction endonuclease²⁹ and sequence³⁰ analyses of these mutant β -globin genes have revealed nucleotide alterations only within the 5' consensus sequence of some of these genes. It is probable that these

β -thalassaemias are associated with disruption of RNA splicing, although this has not been confirmed experimentally.

Models have been advanced suggesting mechanisms for splicing based on hybridizations between small nuclear RNAs and the consensus sequences at the 5' and 3' splice sites of mRNA precursors^{1-3,31}. These proposals are supported by recent evidence that a small nuclear ribonucleoprotein particle is required for splicing of adenovirus early RNAs³². They suggest that failure to splice the pm975 12S mRNA may result from instability of a precursor-small nuclear RNA hybrid. Alternatively, the enzyme(s) involved in the cutting and ligating steps of RNA splicing³³ may have a high degree of specificity for a U in the second position of the intron.

We selected the specific point mutation on the basis of its likelihood to disrupt the 12S mRNA splicing event while leaving the 289 amino acid protein encoded by the 13S mRNA unaltered because of degeneracy in the genetic code. This represents an effective approach with general applicability for ascribing specific functions to individual messages in a series of overlapping spliced RNAs.

Despite the absence of the 12S mRNA, the phenotype of Ad2/5 pm975 on HeLa and HEK cells is indistinguishable from wild-type Ad2 or Ad5, indicating that the only early region 1A function necessary for virus replication in human cells is

encoded in the 13S mRNA. Additionally, these results establish that the EIA regulatory element necessary for modulation of the early adenovirus transcription units is a protein encoded solely by the EIA 13S mRNA. Thus a single mRNA encoding a positive regulatory protein in eukaryotes has been identified.

Despite the apparent dispensability of the 12S mRNA, it probably encodes a function which has been selected over a long evolutionary period. Analysis of EIA mRNAs from serotypically unrelated adenoviruses (Ad 7 and Ad 12) reveals spliced structures similar to the 12S and 13S mRNAs of Ad2^{34,35}. The ubiquity of these mRNAs among various subgroups of adenovirus demonstrates that these structures survived significant evolutionary divergence, which implies that the 12S mRNAs encode a biologically important function *in situ*.

Continued analyses of pm975, including its transforming capacity on primary rodent cells, are underway. The results will reveal whether the same viral protein which modulates early gene expression also induces cellular transformation.

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Unusual physical organization of the *Halobacterium* genome

Carmen Sapienza & W. Ford Doolittle

Department of Biochemistry, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

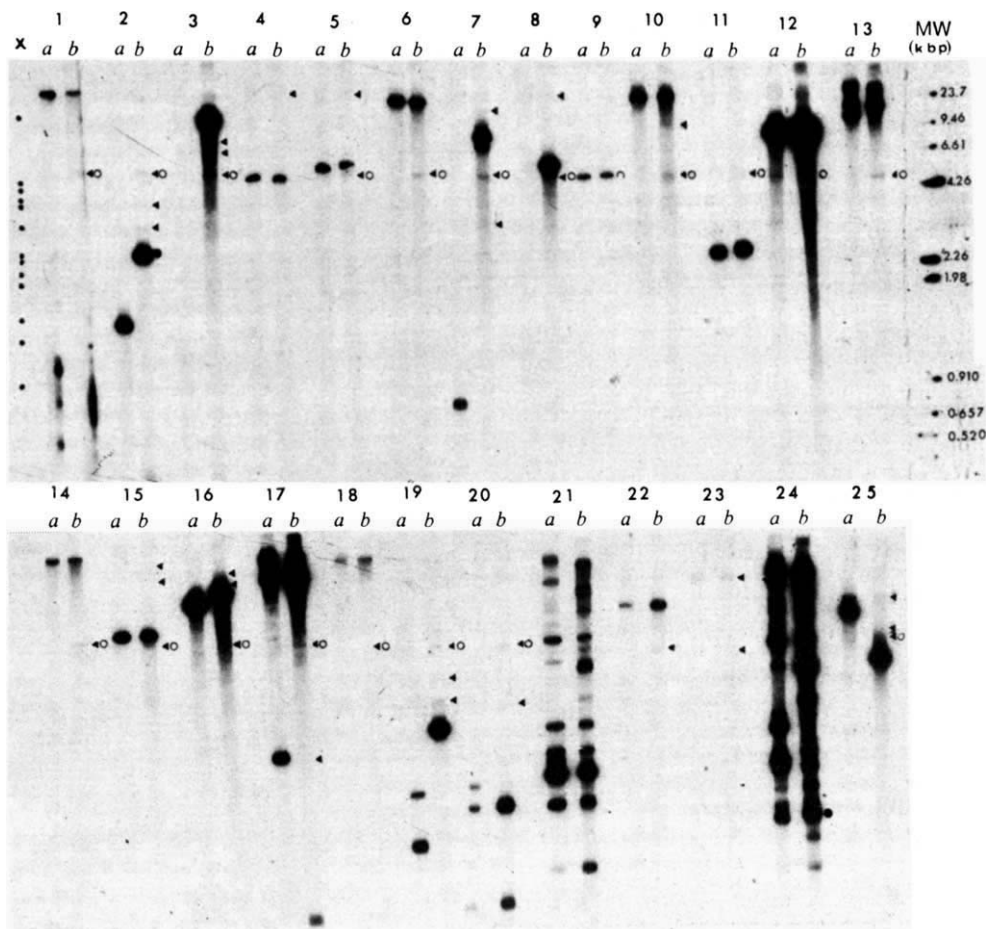
The genomes of the extremely halophilic bacteria, Halobacterium halobium and Halobacterium volcanii, contain many repeated sequences. These sequences comprise many families, seem to be highly mobile and are arranged in both clustered and dispersed fashions within these genomes. At least some repeated sequences are more strongly conserved between the two species than are unique sequence DNAs.

THE archaeobacteria (halobacteria, methanogens and certain thermophiles) are thought to have diverged from eubacteria, and from the lineage which gave rise to the nucleus and cytoplasm of eukaryotic cells, at a very early stage in cellular evolution¹⁻³. The metabolism, physiology and molecular biology of archaeobacteria exhibit some traits which are unique and others which may be considered either 'eubacterial' or 'eukaryotic' in nature¹⁻⁴. Virtually nothing is known about

archaeobacterial genomes except their genetic complexities. That of *Methanobacterium thermoautotrophicum* has a molecular weight of $\sim 1.1 \times 10^9$ (ref. 5), that of *Thermoplasma acidophilum*⁶ $\sim 0.8 \times 10^9$ and those of several halobacteria^{7,8} have molecular weights of 2.5×10^9 .

The DNA of *Halobacterium halobium* and related species can be separated into components of 66-68 and 57-60 mol % G+C (refs 7, 8). Much, but not all, of the latter fraction comprises a

Fig. 1 Autoradiogram showing hybridization of individual ^{32}P -labelled recombinant plasmid DNAs from a genomic library of *H. halobium* R-1 to total DNA from *H. halobium* NRC-1 (tracks a) and *H. halobium* R-1 (tracks b). ◀, Position of faint bands of hybridization; ○, position of an ~5 kbp band probed by many of the cloned DNAs. Large closed circles in tracks 2b and 24b indicate hybridization to plasmid DNA fragments. Small closed circles in track X indicate the positions of *Eco*RI fragments of strain R-1 plasmid DNA. The genomic library was constructed by ligating *Bam*HI-*Eco*RI restriction endonuclease (Boehringer) double-digested *H. halobium* R-1 DNA to similarly cleaved pBR322 using T4 DNA ligase¹⁵ (Boehringer). *E. coli* JF1754 (obtained from J. Friesen) was transformed¹⁶ with the ligation mixture and plasmid DNA isolated¹⁷ from 35 Amp^r Tet^r colonies. The recombinant plasmid DNAs were labelled with [α - ^{32}P]dCTP (NEN) by nick-translation¹¹. *H. halobium* R-1 and NRC-1 DNA was prepared as described elsewhere¹⁸, cleaved with *Eco*RI and subjected to electrophoresis in a 1.2% agarose gel (Sigma). DNA was transferred to a sheet of nitrocellulose filter paper¹² (Millipore) and 0.5 cm strips cut from the sheet. Each probe was hybridized to one 'blot' of each of NRC-1 and R-1 DNA and washed in stringent conditions¹⁹. Molecular weight markers were λ c1857 *Sam*7 DNA digested with *Hind*III and plasmid pBR322 DNA digested with *Alu*I.



150 kilobase pair (kbp) plasmid, four to five copies of which are present in *H. halobium* (ref. 9 and F. Pfeifer, personal communication). Changes in plasmid DNA restriction endonuclease digestion patterns, which can be interpreted as resulting from complex and multiple insertion and deletion events, are associated with easily detectable phenotypic alterations in gas vacuole and pigment production¹⁰. Such alterations occur with astonishing frequency, suggesting that the *Halobacterium* genome may contain many transposable elements or regions of sequence homology promoting recombination in and between chromosomal and plasmid DNAs.

Renaturation kinetic analyses show no substantial rapidly reannealing fraction which might represent the DNAs of a few, high copy number, repeat sequence families^{7,8}. Data presented here, however, do show that (1) the genomes of halobacteria harbour many different, and perhaps rather small, families of repeated sequences whose members are probably both dispersed and clustered in both plasmid and chromosomal DNAs, (2) some repetitive DNAs are more highly conserved in sequence between two distantly related halobacterial species than are unique sequence DNAs, and (3) genomic rearrangements affecting the location of repetitive sequences are frequent and not necessarily associated with detectable phenotypic alterations.

Repeated sequences in a *Bam*HI-*Eco*RI library of *H. halobium* R-1 genomic clones

H. halobium strain R-1 was isolated as a spontaneously occurring, gas vacuole-deficient variant of the 'wild-type' strain NRC-1 in 1969 (W. Stoekenius, personal communication). It harbours a plasmid of ~50 kbp. Total R-1 DNA doubly digested with *Bam*HI and *Eco*RI was ligated into similarly digested pBR322 and used to transform *Escherichia coli* JF1754. Thirty-five Amp^r Tet^r transformants were randomly selected, and their recombinant DNAs were labelled by nick-translation¹¹ with

^{32}P -dCTP and used to probe *Eco*RI-digested strain NRC-1 or strain R-1 DNAs bound to nitrocellulose paper strips¹². Thirty-one of the labelled DNAs probed more than one *Eco*RI fragment of NRC-1 or R-1 DNA; thus each probe contained at least one sequence present more than once in these genomes. Figure 1 shows results for 25 of these cloned probes (faint bands detectable on the original autoradiograph are indicated by ◀). All but clone 9 probe *Eco*RI fragments in addition to those corresponding to the fragment cloned. Sixty to seventy such 'extra fragments' are probed in strain R-1 DNA (tracks b); slightly fewer strain NRC-1 DNA fragments (tracks a) are probed, and many of these have mobilities different from the probed R-1 fragments. Clones 2 and 24 each probe single and different fragments (indicated by large closed circles) with similar mobilities to *Eco*RI fragments of strain R-1 plasmid DNA (data not shown, but positions of plasmid bands indicated by ● in track X); most of the remaining probed fragments are of chromosomal origin. Although no clones seem to show identical probing patterns (considering patterns obtained with both NRC-1 and R-1), all but clones 4, 21, 22, 23 and possibly 24 probe, with varying intensities, a common ~5 kbp chromosomal fragment in R-1 DNA (○) which is not probed in NRC-1 DNA. For four of these clones probing this fragment (clones 6, 11, 14 and 18), it is the only fragment probed in addition to that cloned; the remaining 15 probing this common 5 kbp fragment probe fragments in addition to this and the fragment cloned (except clone 9, which may contain only the 5 kbp fragment). Thus the 5 kbp common fragment must contain copies of many (>10) different elements which are present in one or two locations elsewhere in the genome. This cluster must either have been created in strain R-1, or disrupted in strain NRC-1, since their divergence 12 yr ago.

The 25 clones used for Fig. 1 and the remaining 10 (not shown) contain in total 250 kbp of *H. halobium* DNA, or 6% of the genome, and contain a minimum of 31 different elements present at least once elsewhere in the genome. If they represent

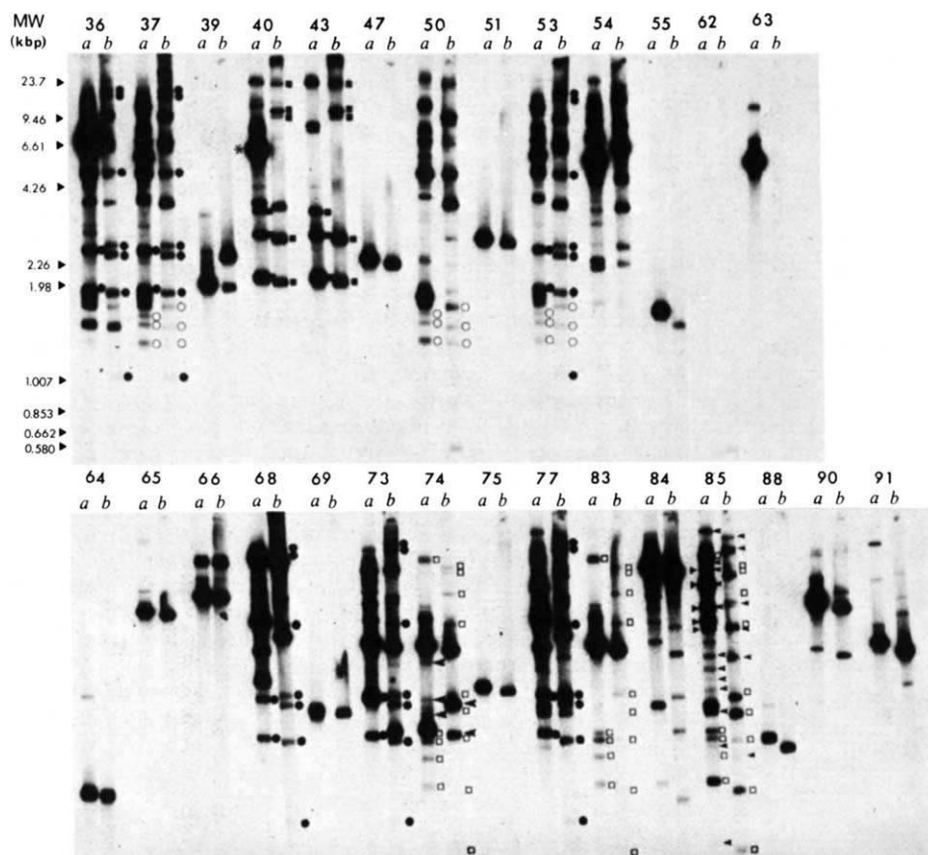


Fig. 2 Autoradiogram showing hybridization of individual ^{32}P -labelled recombinant plasmid DNAs from a genomic library of *H. halobium* NRC-1 to *EcoRI*-digested total DNA from *H. halobium* NRC-1 (tracks a) and *H. halobium* R-1 (tracks b). The library was constructed by ligating *EcoRI*-digested NRC-1 DNA into *EcoRI*-digested pBR322. All experimental procedures were as described in Fig. 1 legend except that both $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ and $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ were included in the nick-translation reaction. Open and closed circles and squares indicate shared subpatterns probed by some of the cloned fragments. Small and large arrowheads indicate fragments which distinguish clones 74 and 85 from each other and from clone 83. Closed triangles adjacent to clone 85 (track a) indicate *EcoRI* fragments of *H. halobium* NRC-1 DNA which are also probed by several *H. volcanii* cloned DNAs (see Fig. 3b).

a random sample of total DNA, there must be at least 500 repeated elements comprising many families in the strain R-1 genome. Some elements must be clustered on cloned DNA, or present in copy numbers >10 in the genome, because clones 21 and 24 alone probe more than one-third of the 'extra' fragments. Variations in intensity of hybridization signals (most obvious with clones 17, 21 and 24) could mean that the fragment probed contains divergent copies of the elements present on the probe, or varying numbers of copies of such elements, or that the probes themselves contain several different repeated elements. The latter interpretation is favoured by data discussed below.

Repeated sequences in an *EcoRI* library of *H. halobium* NRC-1 genomic clones

Strain R-1 is a mutant, and the unknown event which created it may have resulted in the dispersal of repeated sequences within the genome. We constructed a library of wild-type (NRC-1) *EcoRI* fragments cloned into *EcoRI*-digested pBR322 and used 28 of the resulting labelled recombinant DNAs to probe NRC-1 and R-1 DNAs digested with *EcoRI* (Fig. 2). One of these (clone 62) probed only a single fragment of NRC-1 and R-1 DNAs; the rest probed multiple fragments of either NRC-1 or R-1 DNA. As the NRC-1 plasmid is large (150 kbp), not easily isolated and gives a complex *EcoRI* digestion pattern, it is difficult to say how many probed fragments are of plasmid origin. Most of the fragments probed by at least one of these clones (clone 37) are, however, not of plasmid origin, as judged by comparisons of mobilities of probed *EcoRI*-, *HindIII*-, *SalI*-, *SstI*- and *SstII*-generated fragments to plasmid fragments produced by these enzymes (data not shown). All cloned fragments produced different Southern hybridization patterns with DNAs of strains NRC-1 (Fig. 2 tracks a) and R-1 (Fig. 2 tracks b), and all (except for clones 37 and 53, which may be identical) produced different, probe-specific patterns with each of these DNAs. However several fragments are jointly probed, in different combinations, by different cloned fragments, and can be used to identify at least four shared 'subpatterns' (indicated by open and closed circles and squares in Fig. 2) which are

probed in various combinations by clones 36, 37, 40, 43, 50, 53, 68, 73, 74, 77, 83 and 85 (◄ identifies some fragments which distinguish clones 74 and 85 from each other and from clone 83, which otherwise probe common fragments). These 'subpatterns' are also apparent when the same clones are used to probe *PstI*-digested NRC-1 DNA; those probing various sets of *EcoRI* fragments in different characteristic combinations also probe different sets of *PstI* fragments in different characteristic combinations (data not shown). The remaining clones produce hybridization patterns with *EcoRI*-digested NRC-1 DNA which appear unique (and also produce unique patterns with *PstI*-digested DNA). It is not clear whether these patterns too are composed of 'subpatterns', but intensity variations suggest they are. In any case, at least 21 different repetitive sequences are present among these 28 clones (some several times), and most or all of these are different (because they produce different probing patterns) from the repetitive sequences present in clones of the *BamHI*-*EcoRI* strain RI library. Note that clones 40 and 43 probe common fragments and thus must contain a common element(s), and yet the NRC-1 DNA insert in clone 40 (indicated by an * in Fig. 2) is not itself detectably probed by clone 43. Also, some identical fragments probed by clones 50 and 53 (or 83 and 85) produce hybridization signals of different intensities.

Many of the cloned fragments in the *EcoRI* strain NRC-1 library bear copies of different repetitive elements, some of which must lie close to each other in the genome. Cloned fragments which unquestionably bear two different elements (clones 36, 37, 40, 50, 53, 74, 77 and 85) have an average length of only 3.3 kbp (range 1.75–4.6 kbp). We suspect that the differing intensities with which individual fragments are probed by different cloned DNAs reflect the presence of different numbers of copies, on both probed and cloned fragments, of shared sequences.

There should be (on the basis of its G+C content) ~300 *EcoRI* fragments in the *H. halobium* genome. Elements indicated by symbols in Fig. 2 appear to be present on 10 or fewer *EcoRI* fragments, and yet one of these elements appears five or six times and two of them two or three times in our 28

clones. There are two possible explanations for this apparent statistical improbability. (1) Such elements are present in many copies on the few *EcoRI* fragments which are detected as probed, and also present on a much larger number of other *EcoRI* fragments, but in too few copies to give detectable hybridization signals. Clones bearing *EcoRI* fragments of the latter type would then only probe strongly fragments of the former type. The results with clones 40 and 43 noted above might be consistent with this interpretation. (2) The repetitive sequences represented in Fig. 2 are preferentially located in A+T-rich regions of the genome which are more frequently attacked by *EcoRI*. Indeed, most of the cloned fragments are smaller (average length 3 kbp) than the expected distance between *EcoRI* sites (12 kbp). We may be repeatedly sampling only a small portion of the genome.

To test the latter possibility, we constructed a small library of *PstI* fragments of NRC-1 DNA inserted into *PstI*-digested pBR322. The *PstI* recognition site is similar in G+C content to the *H. halobium* genome. Thirteen such cloned DNAs were used to probe *PstI* digests of total DNA of strain NRC-1. None probed fragments other than that corresponding in mobility to the fragment cloned, and yet the 13 clones contained in total 48.7 kbp of *H. halobium* NRC-1 DNA, or 51% of the 94.7 kbp represented by *EcoRI* clones shown in Fig. 2, in which there are at least 30 repetitive elements of at least 21 different families.

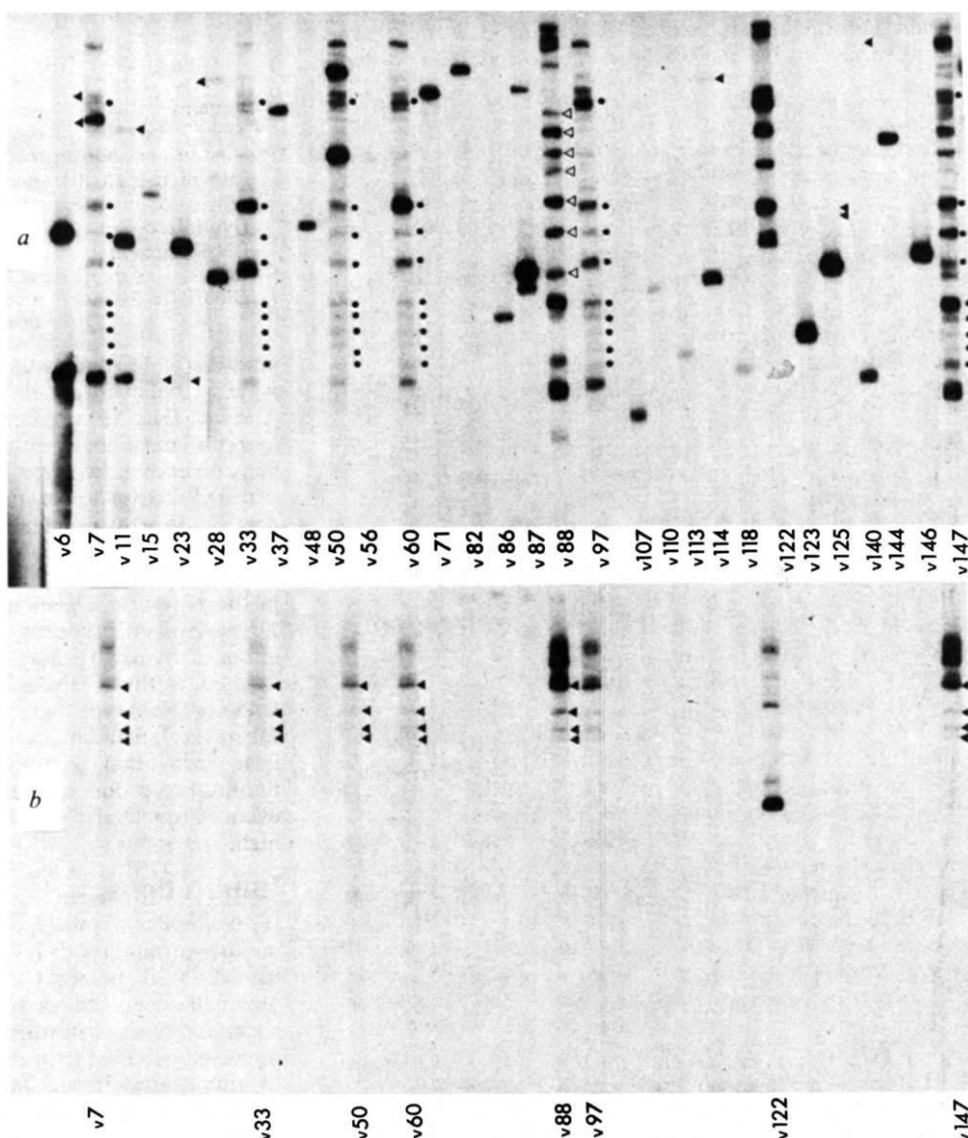
Repeated sequences in *H. volcanii* genome

16S ribosomal RNA (rRNA) catalogue analyses show *H. vol-*

canii to be as remote phylogenetically from *H. halobium* as *Pasteurella* or *Aeromonas* species are from *E. coli*². To determine whether *H. volcanii* also contains families of repeated elements and shares any of these with *H. halobium*, we constructed an *EcoRI* library of *H. volcanii* total DNA and used cloned fragments to probe *EcoRI*-digested *H. volcanii* and *H. halobium* NRC-1 DNAs (Figs 3a, b). Of 30 randomly selected clones, 10 (v71, v82, v86, v107, v110, v113, v118, v123, v144 and v146) contained unique sequence DNA and only probed fragments identical in mobility to the fragment cloned; none of these probed any fragment of NRC-1 DNA. The remaining 20 cloned DNAs probed multiple fragments of *H. volcanii* DNA (the faintest of which are marked by ▲ in Fig. 3a). Five different cloned DNAs (v7, v33, v50, v60 and v97) probed an identical large set of *H. volcanii* fragments (some are indicated by ● in Fig. 3a). Clone v147 probed the same set, plus several additional fragments. The remaining multiply probing cloned fragments produced hybridization patterns which appear unique, although only two of these (v88 and v122) probed more than three or four fragments in addition to that representing the cloned insert. Only those *H. volcanii* clones probing more than three or four *H. volcanii* DNA fragments probed *H. halobium* NRC-1 DNA but each of these probed multiple fragments (Fig. 3b). Thus some repeated sequences are more highly conserved than are unique sequence DNAs.

The clones probing *H. volcanii* fragments indicated by closed circles in Fig. 3a probed multiple and similar fragments of *EcoRI*-digested *H. halobium* NRC-1 DNA. Clone v147, which

Fig. 3 Autoradiogram showing hybridization of individual ³²P-labelled recombinant plasmid DNAs of a genomic library of *H. volcanii* to *EcoRI*-digested total DNA from *H. volcanii* (a) or *H. halobium* NRC-1 (b). The library was constructed by ligating *EcoRI*-digested total *H. volcanii* DNA to similarly digested pBR322. Both [α -³²P]dCTP and [α -³²P]dATP were included in the nick-translation reaction. All other experimental procedures were as for Fig. 1. In a, ▲ indicates faint bands detected on the original autoradiogram; ●, *EcoRI* fragments of *H. volcanii* DNA hybridized by several *H. volcanii* cloned DNAs and by two *H. halobium* cloned DNAs (see Fig. 4); ◀ adjacent to clone v88 indicates *EcoRI* fragments of *H. volcanii* DNA also hybridized by three *H. halobium* cloned DNAs (see Fig. 4). In b, ▲ indicates *EcoRI* fragments of *H. halobium* DNA probed by several *H. volcanii* cloned DNAs and probed by *H. halobium* clone 85 (see Fig. 2).



must contain two different repetitive elements, probed the same fragments and one or two others. Clone v122 probed a unique set of *H. halobium* NRC-1 fragments. More unexpectedly, clone v88 probed the same set of *H. halobium* NRC-1 fragments as did clones v7, v33, v50, v60, v97 and v147, although it does not probe the same set of *H. volcanii* fragments. Thus, repeated elements which are usually separated in *H. volcanii* DNA are usually associated in *H. halobium*.

In a reciprocal experiment (Fig. 4), selected *Eco*RI clones of *H. halobium* NRC-1 DNA which probed multiple *Eco*RI fragments of that DNA were used to probe *Eco*RI-digested *H. volcanii* DNA. Clones 54 and 85 (Fig. 2) probed identical sets of *H. volcanii* DNA fragments, which seem to include all the fragments probed by *H. volcanii* clones v88 and v97 (or others like it), plus some additional high-molecular-weight fragments. This provides reciprocal confirmation of the conclusion reached above. These clones contain inserts of only 3.3 and 1.75 kbp, respectively. However, *H. halobium* NRC-1 clone 84 probes only those fragments probed by *H. volcanii* clone v88, so this clustering is not obligatory in *H. halobium*. Of the remaining seven *H. halobium* NRC-1 clones tested, four (37, 40, 51 and 91) probed unique sets of *H. volcanii* *Eco*RI fragments and three probed no *H. volcanii* fragments.

Rearrangements affecting *H. halobium* repetitive sequences

Figure 5 shows results obtained by probing *Eco*RI (a) or *Sal*I (b) digested DNAs from strain NRC-1 (track 1), two NRC-1 single-colony isolates out of 20 picked from a single plate as phenotypically indistinguishable from NRC-1 (tracks 2 and 3), two spontaneously arising gas vacuole-deficient NRC-1 variants

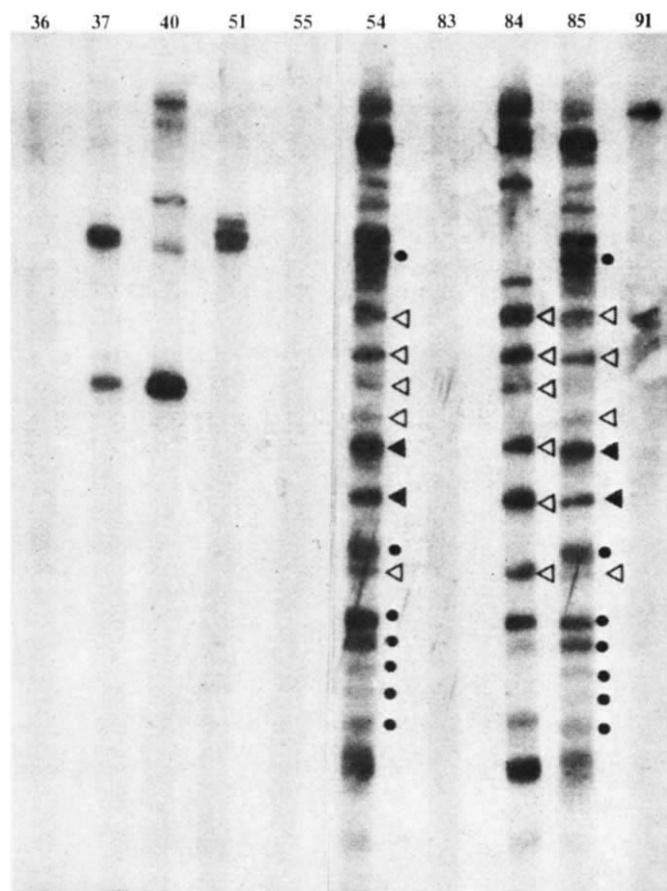


Fig. 4 Autoradiogram showing hybridization of *H. halobium* NRC-1 recombinant plasmid DNAs (see Fig. 2) to *Eco*RI-digested *H. volcanii* DNA. ●, ◁, Fragments also hybridized by *H. volcanii* cloned DNAs (see Fig. 3). ◀, Superimposed symbols ● and △.

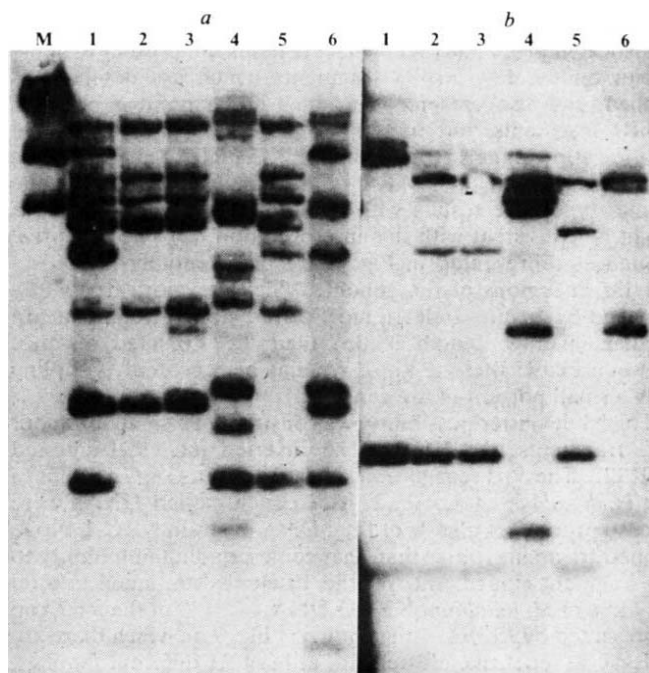


Fig. 5 a, Autoradiogram showing hybridization of a 32 P-labelled 330 bp *Alu*I fragment of *H. halobium* NRC-1 DNA to *Eco*RI-cleaved total DNA from *H. halobium* NRC-1 (lane 1), two apparently wild-type colonies of strain NRC-1 selected from 20 such isolates (see text; lanes 2 and 3), two gas vacuole-deficient mutants of strain NRC-1 (such mutants arise at a frequency of $1-3 \times 10^{-2}$) (lanes 4 and 5), and *H. halobium* R-1 (lane 6). Lane M contains 32 P-labelled λ c1857 *Sam*7 DNA cleaved with *Hind*III. The 330 bp *Alu*I fragment used as a probe appears to comprise most of an ~500 bp repetitive element present in one copy on each of two *Hind*III-*Eco*RI cloned fragments of NRC-1 DNA which do not otherwise cross-hybridize (data not shown). b, Hybridization of a 32 P-labelled 900 bp *Pst*I-*Sal*I strain R-1 cloned DNA fragment to *Sal*I-cleaved DNAs from different *H. halobium* strains. This fragment was discovered as a 900 bp insertion in a *Hind*III fragment of strain R-1 plasmid DNA (see text). Lane designations as in a.

(tracks 4 and 5) and strain R-1 (track 6) with two DNAs bearing what we believe to be different and single repetitive elements. The first (Fig. 5a) is a 330 bp *Alu*I fragment which appears to comprise most of an ~500 bp repetitive element present in one copy on each of two *Hind*III-*Eco*RI fragments of NRC-1 DNA which do not otherwise cross-hybridize (data not shown). The second (Fig. 5b) is a cloned ~900 bp fragment containing an element present on one of two otherwise identical *Hind*III fragments of the *H. halobium* R-1 plasmid. (This element is also present at least a dozen times in the *H. volcanii* genome; data not shown.) None of the six DNAs probed produces identical hybridization patterns with either of these two probes, even though the cell populations used to prepare these DNAs derive from phenotypically identical colonies ('wild type' for tracks 1, 2 and 3; gas vacuole-deficient for tracks 4, 5 and 6) and were (except for strain R1) separated from each other by only the number of generations (~30) required to produce enough cells to prepare DNA. Mobility, even of repeated elements selected randomly, is remarkably high.

Implications

(1) *H. halobium* and *H. volcanii* genomes each contain a large number of families of repeated sequences, each of which may have 2- >20 members. The different hybridization patterns obtained when clones from each of the two *H. halobium* libraries discussed above, and a third *Hind*III-*Eco*RI library not discussed, are used to probe *Eco*RI-digested *H. halobium* DNA identify at least 50 such families, and there may be many more. The possession of multiple repetitive sequence families is not typical of all archaebacteria; comparable cloning and hybri-

dization experiments with the DNA of the much smaller genome of *T. acidophilum* (not shown) revealed no repetitive sequences. (2) Repetitive elements are often clustered together with other repetitive elements of other families within small genomic regions. Elements of a given family may be clustered with elements of different families in different regions. Some clusters may themselves comprise repeat sequence families, but we cannot be certain of this. (3) Some clusters, such as the 5 kbp fragment identified in Fig. 1, may contain quite large numbers of different elements. (4) Rearrangements which affect the disposition of repeated sequences occur very frequently, and are not obligatorily coupled with any detectable phenotypic alteration, although such alterations are themselves very frequent. The high frequencies of both types of event make it difficult to relate them causally¹⁰. (5) Sequences which are repeated in the genome of *H. halobium* are frequently present and repeated in the genome of *H. volcanii* and vice versa. They may often be clustered with elements of different repeat sequences in different ways in the two genomes. Most unique sequence DNAs are not conserved between the two genomes. (6) The

genome may contain regions of non-random variation in G + C content, in which elements of different families are mostly clustered, because libraries prepared with restriction endonucleases recognizing targets of different per cent G + C contain different numbers and kinds of repeated elements. Although the *H. halobium* chromosome and plasmid are of different G + C contents, they do not contain mutually exclusive sets of repetitive elements (Figs 1, 2 and data not shown). Such compartmentalization, if it exists, is a property of the whole genome.

We know of no other data on prokaryotic genomes which reveal the complexity of repeat sequence composition, arrangement and rearrangement demonstrated here. The only detailed body of data of comparable complexity is that bearing on repeated sequences in the genomes of plants¹³ and sea urchins¹⁴, but we hesitate to suggest, on this basis alone, that the halobacterial genome resembles the genomes of eukaryotes more than those of eubacteria. This issue deserves more intensive scrutiny.

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LETTERS TO NATURE

Detection of HC₁₁N in IRC+10°216

M. B. Bell, P. A. Feldman, Sun Kwok
& H. E. Matthews

Herzberg Institute of Astrophysics,
National Research Council of Canada,
Ottawa, Canada K1A 0R6

The observations of three rotational transitions ($J = 70 \rightarrow 69$, $71 \rightarrow 70$, and $72 \rightarrow 71$) of HC₁₁N (cyano-deca-penta-yne) in the microwave emission spectrum of the circumstellar envelope of the cool carbon star IRC+10°216 are reported here. The abundances relative to molecular hydrogen and HC₇N are estimated to be $\sim 7 \times 10^{-8}$ and ~ 0.7 , respectively. With these observations, taken during March and May 1981 at the Haystack Observatory, HC₁₁N becomes the largest and heaviest molecule yet detected outside the Earth's atmosphere.

The cool carbon star IRC+10°216 (=CW Leo) is believed to be undergoing extensive mass loss and its dense circumstellar envelope is known to be rich in neutral molecules and free radicals. Its comparative proximity to the Earth (~ 200 pc) and correspondingly large angular diameter (~ 2.5 arc min as measured in the emission of the CO molecule¹) have made it especially rewarding in this respect.

Observations of the microwave inversion lines of ammonia in the envelope of IRC+10°216 have shown the presence of a weak feature (U23.698) close to the NH₃(1,1) line. The feature appears in observations made at both the 46-m telescope of the Algonquin Radio Observatory² and the 37-m telescope of the Haystack Observatory³. After these observations we tentatively identified U23.698 as the $J = 70 \rightarrow 69$ transition of the linear carbon-chain (cyanopolyne) molecule HC₁₁N (cyano-deca-penta-yne) on the following grounds:

(1) Of the 17 known molecules in the envelope of IRC+10°216, seven have carbon-carbon bonds and 10 have appended CN radicals.

(2) The derived rotational constant ($B_0 \approx 169.27$ MHz) would be only ~ 210 kHz greater than the value predicted by Oka⁴ on the basis of a simple numerical extrapolation from lower cyanopolyne chains. The magnitude ($\sim 0.1\%$) and direction of this discrepancy could be readily explained in terms of a slight molecular non-rigidity which is expected on the basis of the extended vibrational satellite patterns found for carbon-chain molecules⁵.

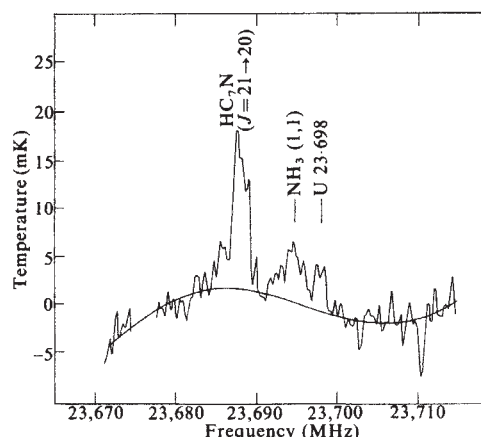


Fig. 1 The spectrum of IRC+10°216 in the region of the NH₃(1,1) line, before baseline removal. Features due to HC₇N ($J = 21 \rightarrow 20$) and the proposed $J = 70 \rightarrow 69$ transition of HC₁₁N (U23.698) also appear in the window. The ordinate is antenna temperature corrected for zenith-angle variations of gain and atmospheric absorption. The gap in the spectrum omits a feature of instrumental origin.

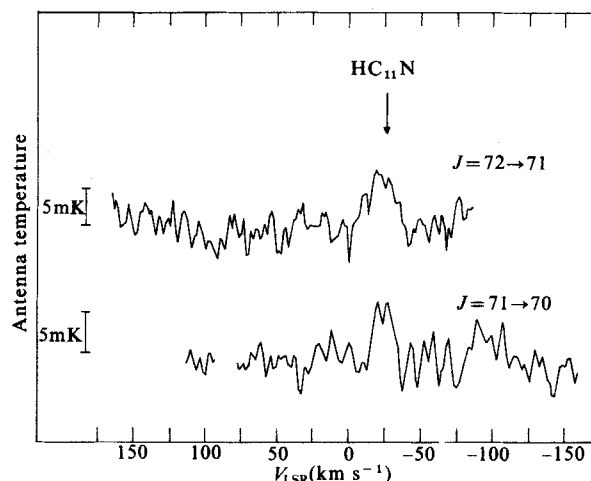


Fig. 2 Two adjacent transitions of HC_{11}N in the spectrum of IRC+10°216. The features have been aligned on a common velocity scale using the rest frequencies given in Table 1. The antenna-temperature scales are indicated on the left of the figure. Other details are as for Fig. 1.

(3) Long-chain cyanopolynes, such as HC_7N , are relatively abundant in IRC+10°216. An extrapolation from the lower members of the molecular series⁶ suggested that $[\text{HC}_{11}\text{N}]/[\text{HC}_7\text{N}] \geq 0.1$. The direct comparison afforded by the presence of both U23.698 and the $J=21 \rightarrow 20$ transition of HC_7N in the $\text{NH}_3(1,1)$ spectrum³ is consistent with this expectation.

We have detected two further microwave transitions of HC_{11}N ($\text{H}-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{C}\equiv\text{N}$) using frequencies derived from the above result. This suggests that the identification of HC_{11}N is fairly secure and now represents the heaviest (147 AMU) and largest ($\sim 15 \text{ \AA}$) extra-terrestrial molecule known.

At the frequencies employed (close to 24 GHz) the 37-m telescope of the Haystack Observatory has a peak aperture efficiency of 22% at 45° elevation and a beam efficiency of 25%, including radome losses. The half-power beamwidth is 1.4 arc min. The observations were carried out with a maser receiver and an autocorrelation spectrometer of total bandwidth 40 MHz and an effective resolution of 4.9 km s^{-1} .

The observations in March were used to search for the $J=72 \rightarrow 71$ transition of HC_{11}N , expected at $\sim 24,374 \text{ MHz}$. The total-power observing method was used; $\sim 25 \text{ h}$ of on-source integration time were accumulated, of which about 30% were affected by adverse weather and were discarded. A further 33 h of on-source time was obtained in May when observations were made of the $J=71 \rightarrow 70$ transition, predicted to be close to 24,037 MHz. On this occasion, measurements were made using a modified 'double-Dicke' mode⁷ when the weather deteriorated, and no data editing was necessary.

Figure 1 shows the spectrum of IRC+10°216 in the region of the $\text{NH}_3(1,1)$ line as obtained at Haystack³. Our new observations of spectral windows containing the proposed $J=71 \rightarrow$

70 and $J=72 \rightarrow 71$ transitions of HC_{11}N are shown in Fig. 2. The arrow indicates the expected position of the line as predicted from the $J=70 \rightarrow 69$ transition first detected at ARO ($B_0 = 169.275 \text{ MHz}$).

Including the earliest measurement² of the $J=70 \rightarrow 69$ transition with the 46-m telescope, four independent observations of microwave rotational transitions of HC_{11}N have been made: the results are summarized in Table 1. The rest frequency has been determined for each case from the data assuming a peculiar velocity for IRC+10°216 with respect to the local standard of rest of -26.0 km s^{-1} (see ref. 1). The $J=70 \rightarrow 69$ transition is confused with the $\text{NH}_3(1,1)$ line and we take the error in determining the rest frequency in this case to be about three times greater than the formal value. The rotational constant B_0 was calculated for each transition using a centrifugal distortion constant $D_0 = 0.22 \text{ Hz}$. The latter was estimated from the semi-empirical relation $D_0(\text{HC}_n\text{N}) \propto [B_0(\text{HC}_n\text{N})]^{2.37}$, which provides a good fit to the measured values of the rotational constants of HCN, HC_3N , HC_5N , and HC_7N , and which is consistent with the published transitions of HC_9N in TMC 1 (refs 8, 9).

The weighted average of B_0 for all four observations is $169.275 \pm 0.001 \text{ MHz}$. This result is unchanged if we omit the poorer-quality 46-m observations of the $J=70 \rightarrow 69$ line, or if we neglect all the $J=70 \rightarrow 69$ data on the grounds of its possible confusion with the $\text{NH}_3(1,1)$ line.

For better estimates of the line parameters we have averaged together the $J=71 \rightarrow 70$ and $J=72 \rightarrow 71$ observations on a common velocity scale (see Fig. 3). The line profile thus obtained may show an indication of the non-gaussian (relatively flat-topped) shape found for other molecules in this source^{1,10}. We derive a full width at half maximum for the line of $25 \pm 2 \text{ km s}^{-1}$ and a peak antenna temperature of $6 \pm 1 \text{ mK}$.

We have detected three lines in the circumstellar envelope of IRC+10°216, which we believe to be adjacent rotational transitions of the long carbon-chain molecule HC_{11}N . While we are confident of the correctness of this assignment, we must still consider whether there are viable alternatives.

The exact agreement in the frequency intervals between the two pairs of lines, and the fact that all three lines have the same intensity leaves little doubt that a single linear species is responsible. If the transitions we detect are not in fact adjacent, but still belong to a single species, that species must have a molecular weight in excess of $\sim 200 \text{ AMU}$. We can argue on abundance grounds that this is much less likely than HC_{11}N .

Certain other linear carbon-chain molecules whose exact line frequencies are not known are expected to have a B_0 of the same order; for example, C_{12}H and C_{11}N . Since C_4H and C_3N are the longest C_nH and C_nN carbon chains of their respective types that have yet been found, and because the derived abundance of HC_{11}N (see below) is in rough agreement with that extrapolated from lower-order cyanopolynes, we are led to identify HC_{11}N as the source of the detected lines.

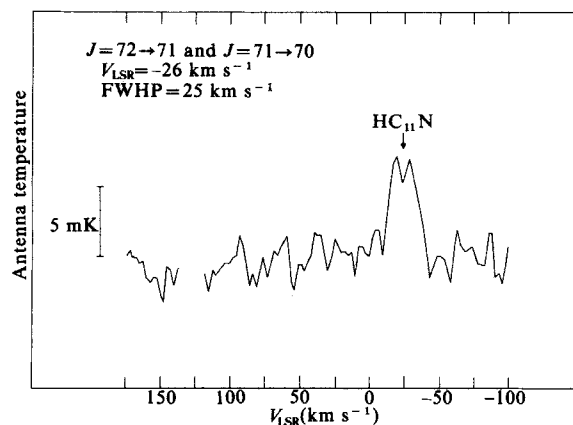


Fig. 3 The average of the $J=72 \rightarrow 71$ and $J=71 \rightarrow 70$ transitions of HC_{11}N in the spectrum of IRC+10°216. Other details are as for Fig. 1.

Table 1 Transitions of HC_{11}N in IRC+10°216

Date	Telescope	Transition $J_u \rightarrow J_l$	Measured rest frequency (MHz)*	Derived B_0 (MHz)†
April 1979	ARO	70 → 69	$23,698.2 \pm 0.3 \ddagger$	169.275 ± 0.002
May 1979	ARO			
February 1980	ARO			
November 1980	Haystack	70 → 69	$23,697.9 \pm 0.4 \ddagger$	169.273 ± 0.003
March 1981	Haystack	72 → 71	$24,375.2 \pm 0.2 \S$	169.274 ± 0.001
May 1981	Haystack	71 → 70	$24,037.1 \pm 0.1 \S$	169.277 ± 0.001

* Peculiar velocity -26.0 km s^{-1} assumed.

† Based on $D_0 = 0.22 \text{ Hz}$; see text.

‡ Line may be confused with $\text{NH}_3(1,1)$; error quoted includes estimate of possible systematic effects.

§ 1σ formal errors.

Table 2 Cyanopolyne abundances in IRC+10°216

Molecule	Observed transition	Observed T_A^* (mK)†	Abundance relative to H_2	
	$J_u \rightarrow J_l$		$\beta = 0.6$	$\beta = 0.8$
$HC_3N^\ddagger$	9 → 8	400 ± 80	7.3×10^{-7}	5.2×10^{-7}
HC_7N	21 → 20	56 ± 8	1.0×10^{-7}	1.0×10^{-7}
$HC_{11}N$	71 → 70	24 ± 4	7.0×10^{-8}	8.0×10^{-8}
	72 → 71			

See text for details of model used.

† Main-beam brightness temperature corrected for atmospheric absorption and telescope gain-zenith angle efficiency variation.

‡ Measured during May 1981 session.

Molecular line excitation in a circumstellar environment has to be treated differently from that in interstellar dust clouds. Not only does the gas density (and thus also collisional excitation) decrease radially outward from the star, but so also does the IR radiation field responsible for the vibrational excitation. Previous radiative transfer models for the CO and NH_3 molecules^{2,11} in the circumstellar envelope of IRC+10°216 show that the excitation temperature gradient can be approximated by a power law of the form $T_{ex}(r) = T_0(r_0/r)^\beta$ where $T_0 = 350$ K at $r_0 = 2 \times 10^{15}$ cm, and $0.6 \leq \beta \leq 0.8$. A gradient of this type, while unimportant for the low rotational states normally observed in the lower-order cyanopolyynes, becomes a significant factor for higher-order members of the series. In the present case, the observed transitions occur between states of rotational energy equivalent to 40 K. The lower excitation temperature in the outer parts of the circumstellar envelope thus leads to a non-uniform distribution of level populations across the molecular gas within the telescope beam.

Model calculations for the circumstellar envelope of IRC+10°216 have been performed assuming the lines are optically thin and the population distribution at each radius to be in LTE at a temperature $T_{ex}(r)$. We further assume that there is neither significant formation nor destruction of the molecules beyond several stellar atmospheric scale heights¹².

The line brightness temperature over the envelope surface is then computed and the results convolved with the telescope beam. Scaling the results to the observed values leads to the molecular abundances (n_{mol}/n_{H_2}) given in Table 2. Note that the derived abundances scale as $(\text{distance}/200 \text{ pc})^2 \times (\text{mass-loss rate}/2 \times 10^{-5} M_\odot \text{ yr}^{-1})^{-1}$ and are uncertain due to the lack of precise values for these parameters. Table 2 also includes results for the $J = 9 \rightarrow 8$ transition of HC_3N , a short observation of which was made in the May 1981 session at Haystack Observatory for comparison purposes. In addition, although we did not observe a transition of HC_9N , we can predict that in IRC+10°216 the HC_9N $J = 41 \rightarrow 40$ line should be approximately twice as strong as the lines of $HC_{11}N$ reported here if $n_{HC_9N}/n_{H_2} = 10^{-7}$.

Thus the abundance of $HC_{11}N$ is ~ 0.7 of that of HC_7N (independent of distance and mass-loss rate). This is interesting in that it is rather higher than would have been expected from an extrapolation from lower-order members of the series. The decrease in abundance as a function of cyanopolyne molecular weight in the envelope of IRC+10°216 also seems to be less steep than in TMC 1 (ref. 8) and other interstellar molecular clouds¹³. Moreover, the cyanopolyne relative abundances compared with molecular hydrogen in IRC+10°216 are much higher than those found in dark molecular clouds. For example, MacLeod *et al.*¹⁴ reported an abundance ratio $[HC_5N]/[H_2] \approx 10^{-8}$ in TMC 1. We find that this ratio is ≈ 60 times higher in the case of IRC+10°216. In fact, relative to H_2 , $HC_{11}N$ is ≈ 7 times more abundant in the envelope of IRC+10°216 than HC_3N is in TMC 1.

Although the exact mechanism for the formation of long-chain carbon molecules in interstellar space is still in controversy¹⁵⁻¹⁸, we now have evidence that large numbers of such molecules can be produced in the atmospheres of carbon stars like IRC+10°216 where activation energies can be overcome¹⁹. Formation of dust grains will quickly lead to momentum grain-gas coupling which will eject the molecules²⁰.

The current rate of mass returned to the interstellar medium by late-type stars is $6 \times 10^{-10} M_\odot \text{ pc}^{-2} \text{ yr}^{-1}$ (ref. 21). Assuming 10% of all late-type stars are capable of manufacturing long-chain carbon molecules, our derived abundances for $HC_{11}N$ implies an ejection rate of 10^{39} $HC_{11}N$ molecules $\text{pc}^{-2} \text{ yr}^{-1}$ into the interstellar medium alone. While most of these molecules will probably be destroyed by interstellar EUV radiation, clearly the molecular production by stars can be an important ingredient in chemical models of the interstellar medium.

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On the reported detection of sub-GeV antiprotons in galactic cosmic rays

David Eichler

Astronomy Program, University of Maryland,
Maryland 20742, USA

Buffington *et al.*¹ claim to have detected a $\bar{p}/p$ ratio of $2.2 \pm 0.6 \times 10^{-4}$ at energies between 130 and 320 MeV. This is several orders of magnitude above theoretical predictions²⁻⁴ at these lower energies for $\bar{p}$ fluxes resulting from collisions of primary cosmic rays with the interstellar gas. Measurements at higher energies by Golden *et al.*⁵ show a $\bar{p}/p$ ratio of $5.2 \pm 1.5 \times 10^{-4}$, in excess, by a factor of about 3, of the expected values if $\bar{p}$ s are of secondary origin in the standard model of galactic cosmic rays. The reported $\bar{He}/He$ ratio¹ is significantly lower than the reported $\bar{p}/p$ ratio, raising difficulties with some scenarios⁶ in which the $\bar{p}$ s are primary cosmic rays from anti-galaxies. Kiraly *et al.*⁷ suggest black hole evaporation as a $\bar{p}$ source. I discuss here scenarios in which the reported sub-GeV $\bar{p}$ excess might be produced as secondaries in high energy collisions. Such production must take place in compact, optically thick sources.

It is assumed that the low energy $\bar{p}$ s have lost a few hundred MeV due to modulation by the solar wind. However, while modulation effects may enhance the $\bar{p}$ excess at low energies, they are not nearly strong enough to account for the claimed value (ref. 7 and R. Jokipii, personal communication) (though such considerations could ease the demands on any model of low energy $\bar{p}$ production). In addition to decelerating cosmic rays,

Present address: Department of Nuclear Physics, Weizmann Institute of Science, Rehovot, Israel.

solar modulation lowers their spatial density so that the low energy $\bar{p}$ flux is significantly greater outside the heliosphere than in the Earth's vicinity.

Kiraly *et al.*⁷ have shown, using Liouville's theorem, that the inferred $\bar{p}$ flux beyond the heliosphere depends very weakly on the assumptions made about modulation. To infer the interstellar number density, N_{Buf} , implied by Buffington *et al.* I assume that the $\bar{p}/p$ ratio they report ($R_{\text{Buf}} = 2.2 \times 10^{-4}$ at ~ 200 MeV) is reached at 600 MeV ($p = 1.2 m_p c$) beyond the heliosphere, and that the integral interstellar momentum spectrum of primaries goes as $p^{-1.7}$. The $\bar{p}/p$ ratio R_{Gol} reported by Golden *et al.* at 7 GeV ($p \approx 8 m_p c$) is 5.2×10^{-4} . Hence, N_{Buf} is given by

$$N_{\text{Buf}} = \left(\frac{8}{1.2}\right)^{1.7} \left(\frac{R_{\text{Buf}}}{R_{\text{Gol}}}\right) N_{\text{Gol}} \approx 10 N_{\text{Gol}} \quad (1)$$

N_{Gol} exceeds the $\bar{p}$ number density N_s predicted by the standard leaky box model of galactic cosmic rays²⁻⁴ by a factor of 3 or so, hence N_{Buf} exceeds that prediction by about one and a half orders of magnitude.

If the $\bar{p}$ excess is real, and if the $\bar{p}$ s are secondaries, they are apparently decelerated outside the heliosphere from their production energy (≈ 3 GeV) down to 600 MeV or so. Scenarios for decelerating secondary $\bar{p}$ s can be broken down into two categories: (1) the $\bar{p}$ s are produced at large cosmological redshift, and are decelerated by the expansion of the Universe; (2) the $\bar{p}$ s are produced and decelerated within the Galaxy. Such scenarios have difficulties with overproduction of secondary products which leads to a model in which the $\bar{p}$ s are produced in dense, optically thick environments—perhaps the galactic centre.

If the $\bar{p}$ s were produced at high redshift, their present cosmic energy density (which would then equal that inside our Galaxy) implied by Buffington *et al.* would be $\sim 10^{-4}$ eV cm⁻³. As the $\bar{p}/\nu$ ratio in high energy collisions is $< 10^{-2}$, the implied energy density in high energy ν s would exceed 10^{-2} eV cm⁻³. (The low γ -ray background immediately constrains the $\bar{p}$ s to be produced in dense compact regions where γ rays would be stopped by collisions with other photons.) The Reines experiment⁸ limits the cosmic neutrino background at $E > 0.5$ GeV to $< 3 \times 10^{-3}$ eV cm⁻³, (ref. 9), apparently ruling out the cosmological deceleration scenario.

Stochastic diffusion in momentum space is one possible mechanism¹ for generating low-energy $\bar{p}$ s. However, such a process would cause the cosmic ray spectrum to approach the equipartition differential momentum spectrum $N(p)dp \propto p^2 dp$, which differs vastly from the observed spectrum $N(p) \propto p^{-2.7}$.

We henceforth consider only adiabatic expansion as a deceleration mechanism. Assume that there is some 'new' cosmic ray population that traverses much matter within a compact expanding region. To produce the reported $\bar{p}$ flux without adding significantly to the observed secondary to primary ratio, (1) the product of the galactic power output P_N of the new population and the traversed grammage g_n must be at most comparable with that of the 'standard' CR population $P_s g_s$ or (2) the new population must traverse enough grammage near the production site for the secondaries to be thoroughly broken down. 50 g cm^{-2} , roughly 5 destruction pathlengths for L, Be and B, suffices¹⁰. Note, however, that at grammage larger than 50 g cm^{-2} , the $\bar{p}$ s begin to annihilate before escaping the compact region. Hence this means of avoiding too much secondary production requires 'fine tuning' of the traversed grammage.

A more general constraint is the γ -ray emission from the galaxy above 100 MeV, $8 \times 10^{38} \text{ erg s}^{-1}$. To within a factor of 2, this is what is expected from the standard CR population interacting with 5 g cm^{-2} of matter before escape¹¹. It would seem, then, that the total collision rate involving galactic cosmic ray protons and the resulting $\bar{p}$ production rate cannot be much greater, in optically thin regions, than expected from the standard cosmic population aside from 'factor of 2' uncertainties. If

$N_{\text{Buf}} \gg N_s$, this poses difficulties for models that would produce the $\bar{p}$ excess claimed by Buffington *et al.* in regions from which γ rays escape.

The γ -ray constraint could be circumvented by postulating $\bar{p}$ production in very young supernova remnants ($T < 10$ yr), for then the associated γ -ray production is sporadic, and could presently be in an 'off' stage. (In any case, in order that primaries produced within the young remnant traverse many g cm^{-2} , it is easy to show that they must do so when the remnant is younger than 10 yr.) This, however, implies that the $\bar{p}$ s suffer enormous adiabatic losses before being deposited in the interstellar medium, and in turn raises the total cosmic ray power and time averaged γ -ray luminosity required to account for the observed $\bar{p}$ flux by an enormous factor. It can be shown that young supernova remnants in nearby galaxies would then be bright γ -ray sources, contrary to observation.

Another constraint on $\bar{p}$ -production comes from the e^+ flux. This constraint is independent of extensive galactic parameters as the $\bar{p}/e^+$ yield depends only on the physics of the high energy collisions. While the results of Buffington *et al.* and Golden *et al.*, given the experimental numbers and other uncertainties, do not necessarily imply too large a $\bar{p}/e^+$ yield in the galaxy, it should be noted that a very large high energy collision rate in the galaxy must be arranged without affecting the observed e^+ flux appreciably. (Possibly, the e^+ s can be disposed of at $E > 1$ GeV by synchrotron radiation in the enhanced magnetic field of a dense environment, if they are produced there.)

In view of the indications that the galactic $\bar{p}/\gamma$ production ratio (and possibly the $\bar{p}/e^+$ ratio) is higher in our Galaxy than expected from cosmic ray collisions, it is worth considering $\bar{p}$ production scenarios that yield such high $\bar{p}/e^+$ and $\bar{p}/\gamma$ ratios. In particular, suppose the anti-protons are produced in dense, compact regions where the radiation density is sufficiently high to block γ -ray escape through photon-photon collisions and to degrade e^+ s through inverse-Compton reactions at a faster rate than $\bar{p}$ annihilation. If the $\bar{p}$ s are produced within ejected fluid, they can be adiabatically cooled, and ultimately deposited into the interstellar medium unaccompanied by π^0 -decay γ rays or $\pi^\pm$ -decay e^+ s.

γ rays at $E_\gamma \approx 100$ MeV are blocked most effectively by X rays at $10 < E_x < 100$ keV. In any compact source the number density n_{ph} of photons of energy E_{ph} is at least $L/4\pi R^2 c E_{\text{ph}}$, where L is the luminosity in these photons and R is the source radius. The source interior is then opaque to γ rays ($\sigma_{\gamma\gamma} n_{\text{ph}} R > 1$) if

$$(L/L_{\text{ed}})(R_s/R)(\sigma_{\gamma\gamma}/\sigma_T)(m_p c^2/E_{\text{ph}}) > 1 \quad (2)$$

Here σ_T and $\sigma_{\gamma\gamma}$ are the cross-sections for Thompson scattering and pair production, R_s is the Schwarzschild radius, and L_{ed} is the Eddington luminosity defined by $L_{\text{ed}} = 4\pi G M m_p c / \sigma_T$, where M is the mass of the compact object and m_p is the proton mass. For $E_\gamma > 100$ MeV, $10 \leq E_{\text{ph}} \leq 100$ keV, this condition is satisfied by accreting compact X-ray sources by a very large margin. (The X rays need not escape; even if they are eventually blocked and converted into fluid motion, they can still block γ rays close to the compact object.) Indeed, high-energy collisions can be postulated to exist near any compact object where $L/L_{\text{ed}} \geq 1$, independent of its mass, without running into the difficulty of overproducing γ rays. Primary high energy $\bar{p}$ s can be produced (say, by collisionless shocks) within the accretion or ejection flow near the object, but the particular mechanism for producing them is not crucial to the model.

The column density in Eddington-limited flow onto any compact object is known, by familiar dimensional arguments, to be of the order of 1 g cm^{-2} or more, independent of mass: $L \approx L_{\text{ed}}$, $L \approx \epsilon \dot{M} c^2$, where $\dot{M}$ is the mass accretion rate, $\epsilon \leq R_s/R$, $\dot{M} \leq 4\pi n m_p R^2 \beta c$ where n is the number density of the accreting material and βc is the radial infall velocity, all of which imply that the column density $n m_p R \approx m_p / \beta \sigma_T > 1 \text{ g cm}^{-2}$. Hence any high energy particles produced in the vicinity of compact objects are likely to have a substantial amount of target matter to interact with.

Finally, if the object ejects matter in the manner of active galactic nuclei or SS433, it can adiabatically decelerate $\bar{p}$ s produced in its vicinity and deposit them into the interstellar medium.

The above scenario also predicts low energy e^+e^- pairs and photons with $E_{ph} \geq 100$ keV from the electromagnetic cascade, and has been proposed¹² to account for the pair production at the galactic centre that is implied by the observed e^+e^- annihilation radiation¹³. The variable 0.51-MeV line emission from the galactic centre requires $\sim 10^{38}$ erg s⁻¹ in pair production if the pairs each have a few MeV in energy, and, according to this scenario, probably requires more than $\sim 10^{39}$ erg s⁻¹ in relativistic primary production. Within an optically thick medium, the $\bar{p}/p$ ratio can approach 2×10^{-3} , so a $\bar{p}$ output of 2×10^{36} erg s⁻¹ or more is reasonable for such a source. This compares favourably with the product of the galactic primary output, 3×10^{40} erg s⁻¹, and the $\bar{p}/p$ ratio measured by Buffington *et al.* of $\sim 2 \times 10^{-4}$. A single source of the sort postulated here, or a few of them, could readily account for the galactic $\bar{p}$ output on energetic grounds. (Note that a necessary condition for producing an e^+e^- annihilation line is the

presence of enough ambient matter so that any e^+ s produced within annihilate. It has been suggested¹⁴ that the clouds near the galactic centre are good candidate sites for the annihilation that separates the observed line. Thus, even if the galactic centre, where the ambient density is very high, proves to be a unique source of e^+e^- annihilation radiation, the phenomenon that produces the e^+ s may be a common one. Other hard X-ray sources, for example, remain possible e^+ and $\bar{p}$ sources through the cascade mechanism even if they display no annihilation line.)

10^{36} erg s⁻¹ in high energy neutrinos at a distance of 10 kpc is detectable by DUMAND, so neutrino astronomy might eventually provide independent evidence for such a $\bar{p}$ scenario.

While the results of Buffington *et al.* may be explainable, they are surprising nonetheless, and independent experimental confirmation is needed. I have also emphasized that the $\bar{p}/e^+$ and $\bar{p}/\gamma$ flux ratios are more important than the $\bar{p}$ flux itself in setting constraints on models of $\bar{p}$ origin.

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Vortex solitary waves in a rotating, turbulent flow

E. J. Hopfinger

Institut de Mécanique (associé au CNRS) Université de Grenoble, Grenoble, 38041 France

F. K. Browand

Department of Aerospace Engineering, University of Southern California, Los Angeles, California 90007, USA

Turbulent flow in a rotating container has been widely studied¹⁻⁵, but here we consider an experiment in which the axis of rotation is vertical, the tank deep and a vigorous turbulence is produced near the bottom by a horizontal grid oscillating in the vertical direction. The flow within the rotating container can be envisioned to result from: (1) the disorganized, turbulent motion produced by the grid which, in the absence of rotation, would yield a random field with turbulent intensity decreasing upwards (away from the grid) and turbulent length scale increasing upwards⁶; (2) the organizing influence of rotation which, in the absence of turbulence, would generate a (trivial) flow having a single vertical component of vorticity, 2Ω (Ω is the tank rotation rate), uniform throughout the tank. These two competing effects combined produce a flow field which is surprisingly complex. The major feature of the flow consists of a collection of regions of highly concentrated vorticity, or vortices. These vortices extend coherently throughout the depth of the container. Waves having the form of compact, helical distortions propagate along the vortex cores. We show that the waves are well described by the vortex soliton theory of Hasimoto⁷.

The Rossby number, $Ro = u/2\Omega l$, measures the local importance of inertia to rotation in the flow field. Both u , the turbulent velocity, and l , the turbulent length scale, can be assumed constant in planes perpendicular to the axis of rotation but, of course, vary with depth. The Rossby number at the grid location is fixed by the motion of the grid itself, and is given by $Ro_g = n/2\Omega$, where n is the grid oscillation frequency. In our

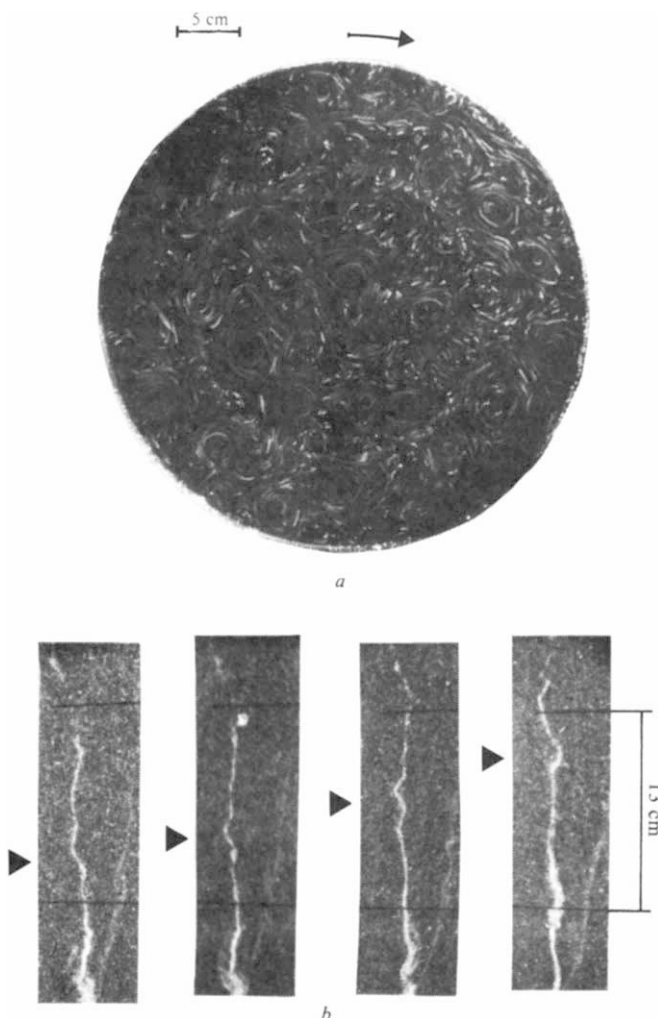


Fig. 1 *a*, Streak photograph illustrating flow in a horizontal plane, $Ro_g = 3.32$. Exposure time is 0.25 s. *b*, Vertical plane visualization of a typical vortex core, utilizing small gas bubbles for illumination. A travelling vortex core wave is marked by arrows.

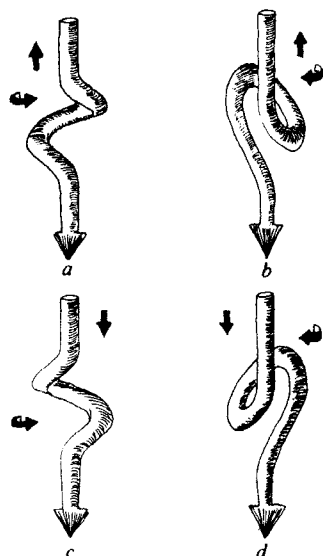


Fig. 2 Schematic representation of possible vortex core wave types. Core vorticity is clockwise, and arrows indicate propagation/rotation of wave patterns.

experiment Ro_g is large, 0(3–30), and the turbulent flow near the grid is relatively unaffected by rotation. Away from the grid the influence of rotation becomes more important, and at some well defined height the flow changes character completely. A collection of concentrated vortices fill the remainder of the tank depth. These vortices are seen in Fig. 1a for a grid Rossby number of 3.32. Here the flow in horizontal planes is visualized by passing a light sheet through the tank and observing the trajectories of small wood particles. The local Rossby number associated with the transition height (and with the vortices above) has a constant value of about 0.20 for the range of grid Rossby numbers investigated. Above the transition, there is almost no additional vertical variation of average turbulence quantities.

For a better visualization of these regions of concentrated vorticity, a vertical light sheet is utilized. Small gas bubbles introduced at the bottom of the tank collect in the core regions to provide the illumination seen in Fig. 1b. (The bubbles themselves do not affect the flow significantly, as the same motions are observed when bubble densities are very low.) Only a fraction of the tank depth is shown, but the vortex cores extend to the top of the tank. The lower ends of the cores terminate in the strongly three-dimensional region near the grid. Quantitative estimates show that a typical vortex has a vorticity 40 times the tank vorticity (2Ω), and a core radius $\epsilon = 0.1$ – 0.15 cm—a remarkable and unexpected result. A complete description of experimental results can be found in ref. 8. Here we concentrate on one interesting consequence of the presence of these vortex regions—the existence of travelling wave disturbances.

A wave disturbance (marked by arrows) can be seen on the vortex core in Fig. 1b. The wave disturbance has the form of a helical distortion of the core. A straight vortex induces no motion on itself, but the wave-distorted core produces a self-induced velocity field which allows propagation of the disturbance. (The propagation of a smoke ring is a simpler example of self-induced motion. The induced velocity field is analogous to the magnetic field which would be produced were the vortex a current-carrying conductor.) The possibility of such vortex core motions was discussed in general by Batchelor⁵ and by Hasimoto⁷. Simple dynamical arguments suggest four possible wave types, as outlined in Fig. 2. Waves *a* and *b* are a left-handed kink and anti-kink, respectively, and propagate in a direction opposite to the core vorticity vector. The kink pattern rotates oppositely to the rotation of the core, while the anti-kink rotates in the same sense as core rotation. Waves *c* and *d* are mirror images of *a* and *b*. All these wave types were observed in our experiment.

The waves appear to propagate as single entities and to travel many times their length without appreciable change in shape. This suggests the possibility of a solitary wave (soliton) descrip-

tion for the phenomenon. Hasimoto⁷ has demonstrated analytically that solitons can exist on a single, isolated vortex core in an irrotational flow. Wave properties are governed by a non-linear Schrödinger equation for a model which assumes constant circulation and no vortex stretching. The induced motion is calculated as if the vortex filament were a circular ring having the same curvature as the actual filament. For a filament which is straight at infinity, there is a particular solution giving a soliton of constant torsion and varying curvature (torsion is the rate of change of bi-normal direction along the filament).

The assumptions of the theory are not precisely appropriate to the present experiment. There are many cores in the experiment, not just a single core; the outer flow is not irrotational; and there is rotation of the container as a whole. These complications seem to be relatively unimportant, however. Individual vortex cores are often far from neighbouring cores, and the concentration of core vorticity by a factor of 40 above the tank vorticity may qualify them approximately as 'isolated' vortices. Finally, Rossby numbers associated with the wave-induced motions are moderately large, that is, $Ro = (\text{group velocity})/2\Omega(\text{wave amplitude}) > 1$. Rotation, then, is responsible for the regions of concentrated vorticity, but has little direct effect on the waves themselves.

The wave chosen for detailed comparison is shown sequentially in Fig. 1b, travelling upwards with anticlockwise rotation (a left kink). The wave length is ~ 5 cm, and the total lateral excursion of the core is ~ 0.6 cm. The corresponding tank rotation rate is 1 revolution per second and the grid oscillation frequency is 6.63 Hz. Two simple comparisons with theory are possible—wave shape and the ratio of propagation speed along the core to the rotation speed of the pattern. The theoretical wave shape predicted by Hasimoto (in suitably non-dimensionalized coordinates) depends on a single parameter,

$$T = \frac{2(\text{Wave torsion})}{(\text{Maximum wave curvature})}$$

The quantity used to non-dimensionalize the physical lengths is

$$\nu = \frac{1}{2} (\text{Maximum wave curvature})$$

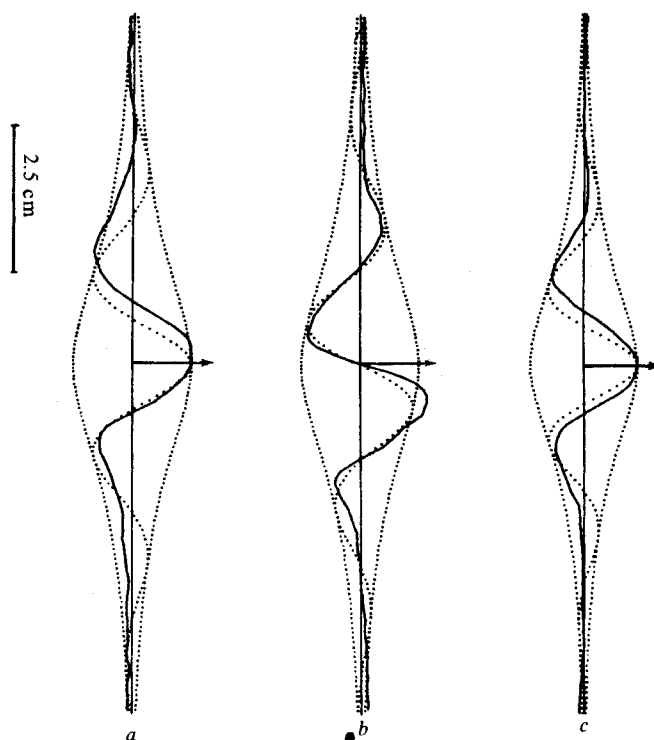


Fig. 3 Comparison of experimental (—) and theoretical (···) soliton wave shapes. *a*, $t = 0$; *b*, $t = \text{one-quarter period}$; *c*, $t = \text{one-half period}$. Lateral scale has been doubled.

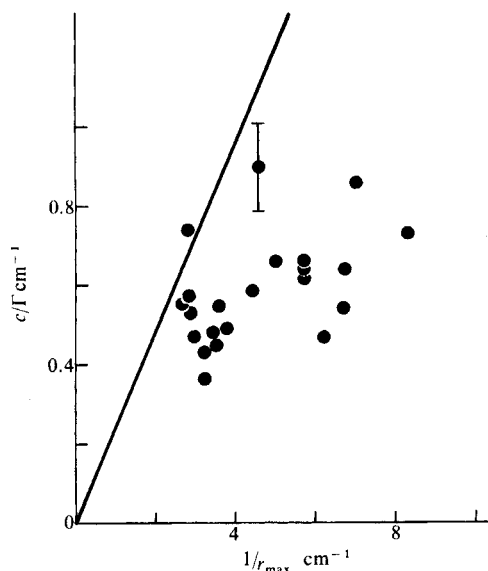


Fig. 4 Observations of group velocity as a function of the inverse of maximum wave radius (taken from film). Solid line is the theoretical upper bound. Error bar indicates relative level of confidence.

In practice, T and ν are chosen to best fit the overall wave shape at three instances of time. The three wave shapes are spaced in time at precisely one-quarter of the pattern revolution, and together represent one-half revolution of the wave. The result of the comparison is shown in Fig. 3. Best overall fit for the three shapes yields the values $T = 3.125$, $\nu = 0.64 \text{ cm}^{-1}$. The wave torsion and maximum curvature are then computed to be, respectively, 2.00 cm^{-1} and 1.28 cm^{-1} . The theoretical envelope is a hyperbolic secant, and the wave shape is a sinusoidal function within the envelope. Theory predicts additional oscillations at the ends of the wave which are not observed experimentally, and this is the most noticeable discrepancy. However, these oscillations are extremely small— $<0.1 \text{ cm}$ —and are probably below the resolution of our visual measurements. Overall, the match between experiment and theory is exceedingly good.

The wave envelope travels upwards with group velocity $c = 15.7 \text{ cm s}^{-1} \pm 10\%$, and has a rotation rate $\omega = 13.2 \text{ rad s}^{-1} \pm 10\%$. Using the observed maximum radius of 0.29 cm , the ratio

$$\frac{\text{Wave envelope speed}}{\text{Maximum rotation speed}} = \frac{c}{\omega r_{\max}} \approx 4.05 \pm 0.7$$

The theoretical prediction is

$$\left| \frac{c}{\omega r_{\max}} \right| = \left| \frac{T(T^2 + 1)}{(T^2 - 1)} \right| = 3.84$$

for $T = 3.125$. Thus, the theoretical estimate agrees with measurement within the accuracy of the measurement. The tentative conclusion is that these isolated waves are indeed solitons and are well described by the theory of Hasimoto.

The theory can be used to draw several additional conclusions in qualitative agreement with experimental observation. The theoretical prediction for the group velocity is

$$c = K \left[\frac{2T}{(T^2 + 1)} \frac{1}{r_{\max}} \right] \Gamma$$

The quantity in parentheses is the wave torsion, here given in terms of T and the maximum wave excursion (or amplitude), $r_{\max}$. Γ is the vortex circulation and K is a constant related to the asymptotic, local-induction approximation. The wave properties just determined, together with an estimated value for the

vortex circulation, allow a rough estimate for the constant K . Thus

$$c \approx 0.48 \frac{T}{(T^2 + 1)} \frac{\Gamma}{r_{\max}}$$

The conclusion is that waves of fixed amplitude, on cores of fixed circulation, travel at different speeds depending on wave shape. Furthermore, waves of fixed shape and fixed circulation travel at a speed inversely proportional to wave amplitude. The reason is that the group velocity is proportional to wave torsion (not to wave amplitude); and for fixed shape, a wave of smaller amplitude has the greater torsion. An approximate upper bound on group velocity is obtained by noting that the quantity $T/(1 + T^2)$ has a maximum value equal to $1/2$ at the value $T = 1$. This value gives

$$c \leq 0.24 \frac{\Gamma}{r_{\max}}$$

for waves on a core of circulation Γ , and having a maximum radial excursion $r_{\max}$. All group velocity measurements should lie below this estimate. Group velocities taken from films are shown in Fig. 4, divided by the estimated value of vortex circulation, $\Gamma = 32.7 \text{ cm}^2 \text{ s}^{-1}$, and plotted against the inverse of the observed maximum wave radius. There is an indication that smaller waves travel faster, but the data are far from conclusive. The upper bound estimate is reasonable for the larger waves, but much too high for waves of smaller amplitude. Possibly other effects limit the propagation velocities of the smallest waves. Note also the relatively small range of wave amplitudes observed. In terms of estimates of the core radius, the largest waves have $r_{\max} = (3-4)\epsilon$ and the smallest waves $r_{\max} \approx \epsilon$.

Our rotating turbulent flow thus consists of a three-dimensional turbulent region near the forcing grid, and a quasi two-dimensional field of strong vortices farther away. Non-linear solitary waves provide the connecting link, and carry energy upwards from the strongly turbulent region. However, waves are also produced along the entire length of the vortex core. This local wave production may provide the vertical stretching and the concomitant horizontal flow convergence necessary to maintain the regions of high vorticity.

Concentrated vortices occur in external flows over wing surfaces and internally in rotating machinery and combustors. Solitary waves are present in these flows and can be seen, for example, during the break-up of wing tip vortices in the wakes of high flying aircraft. The most highly concentrated vortex found in nature is the tornado. Pulsations in core size, rapid vertical motions within the core region and various travelling (wave-like) core deformations have been observed over many years (see refs 10,11). It is plausible that some of these effects are manifestations of vortex core solitons. Solitary waves may indeed be a ubiquitous feature of most turbulent flows which contain concentrated vorticity. The contribution these waves make to turbulent vortex dynamics in complex flows would be a fascinating topic for future study.

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A suspended sediment front in the Severn Estuary

R. Kirby & W. R. Parker

Institute of Oceanographic Sciences, Crossway, Taunton, Somerset TA1 2DW, UK

A zone of marked local gradient in the regional suspended solids field has been located along the axis of the Severn Estuary. This front occupies virtually the same position on ebb and flood and on spring and neap tides, although the amplitude of the gradient fluctuates. Thermohaline fronts have been described in coastal waters^{1,2} suspended sediment fronts associated with the discharge of major rivers into the sea are common³⁻⁵ whilst fronts caused by convergence of secondary circulations within estuaries have also been reported^{6,7}. The previous work describes features with significant differences in the salinity or temperature fields in which any suspended sediment contrasts present are incidental and do not, of themselves, produce large horizontal density variations. In contrast, in the Severn, strong thermohaline gradients in the vicinity of the suspended solids front have not been detected. The mechanisms for the formation and maintenance of the front are not yet known.

The Severn Estuary (Fig. 1) has a large and variable tidal range (12.3 m mean spring range and 6.5 m mean neap range at Avonmouth). It is well mixed with regard to salinity and temperature⁸ but commonly stratified with respect to suspended solids concentration⁹. The suspended load over the greater part of the water column ranges between 0.25 and $>10 \text{ g l}^{-1}$ and its vertical distribution is controlled by the semi-diurnal and spring-neap tidal energy cycles. At maximum flow on spring tides the suspended load is mixed throughout the water column, whereas near slack water on springs and throughout the neap tides, considerable stratification is apparent with concentration levels in the lower layers well in excess of 10.0 g l^{-1} (ref. 9).

The structure of the suspended solids field was observed using a free-fall array consisting of two narrow-gap optical turbidity meters, a pressure (depth) sensor and, occasionally, a continuously recording conductivity meter, allowing continuous and

rapid (1 m s^{-1}) vertical profiles to be made from drifting or anchored vessels. Laboratory calibrations using indigenous material in the range $0-20 \text{ g l}^{-1}$ were later corrected with field samples. Performance curves for the turbidity meters were prepared and they were only used within the ranges where errors were $<10\%$. Over the range of concentration values for which the instruments were calibrated they were known to respond to changes in mass concentration and to be insignificantly affected by changes in the state of aggregation of the solids (T. J. Smith personal communication).

Measurements from anchored vessels have been placed in a regional context during wide ranging surveys of the suspended solids field in the area, resulting in 3,500 vertical profiles from 210 stations based on 17 estuary cross-sections, (standard lines), extending from Watchet to The Shoots (Fig. 1). The regional measurements were made chiefly between 1973 and 1979 on all ranges of tide from spring to neap and during most seasons.

Most of the data have been obtained as time series of cross-sections in which the survey vessel repeatedly traversed a standard line stopping at each station to measure a vertical profile. Between stations the array was towed 2-3 m below the surface and a continuous horizontal traverse was recorded. One complete traverse can be made in approximately 1-1.5 h, in a time series continuing over a 12.5-h semi-diurnal tidal cycle.

The regional surface and bed suspended solids distributions on spring and neap tides was studied by grouping the analogue records into spring (all data $>9.0 \text{ m}$ range) and neap (all data $<9.0 \text{ m}$ range) tides. For most stations this resulted in a minimum of 10 profiles but the limited accessibility of shallow areas resulted in a poor coverage. The surface and bed concentration values for these groups were averaged. The regional spring surface and spring bed distributions (Fig. 2) show the front as a zone of marked gradient (from <0.5 to $>4.0 \text{ g l}^{-1}$ at the bed in 2 km or less) approximately along the axis of the estuary between The Shoots and the Holm Islands, and extending across the inner Bristol Channel to the English coast in the region of Watchet, in Bridgwater Bay. The more turbid water mass lies on the English side of the estuary. On neap tides the regional distribution is similar, although the contrast between surface and bed concentration values is greater.

For more detailed analyses, data from each cross-section have been grouped into spring ebb and flood and neap ebb and flood conditions and the average concentration calculated for each

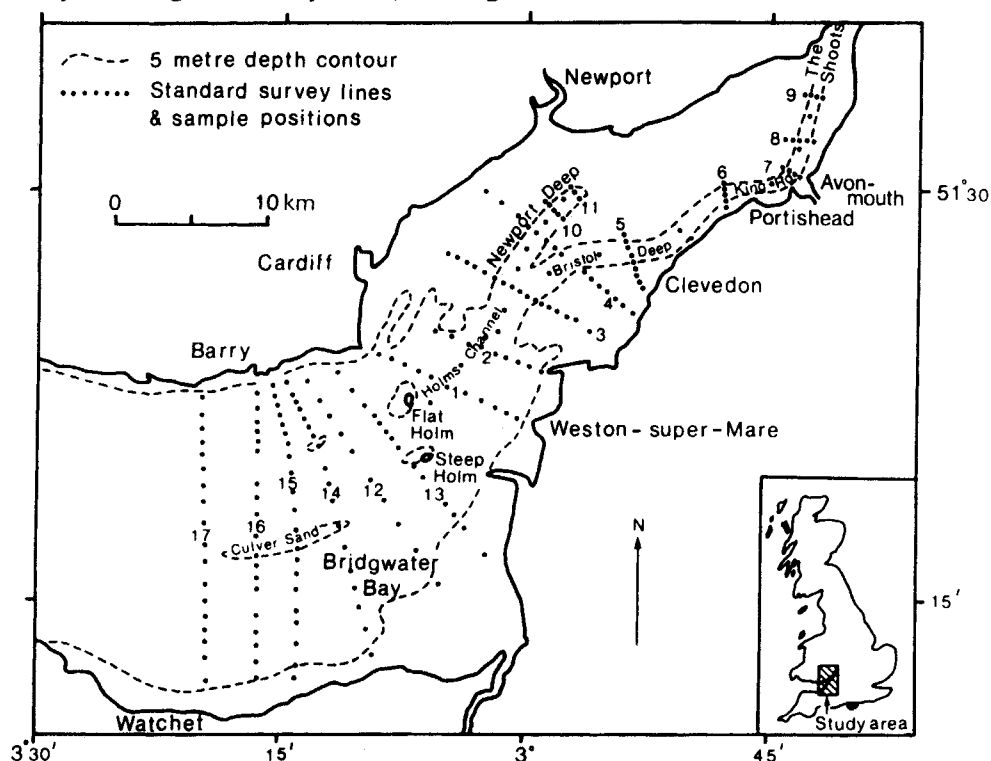


Fig. 1 Location of standard stations used in surveys of suspended solids distribution.

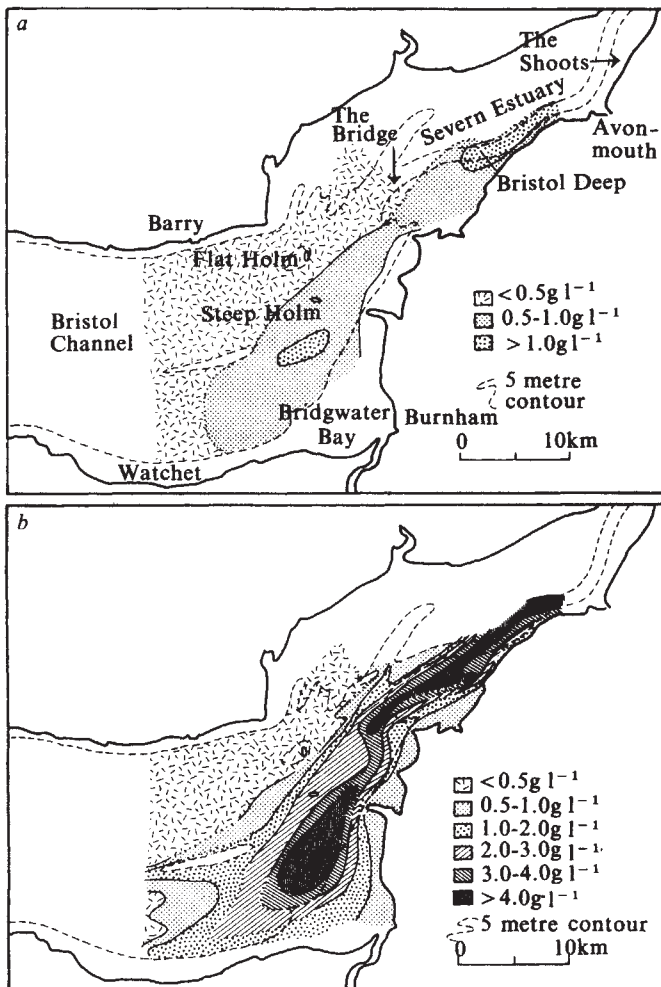


Fig. 2 Plan distributions of spring surface average (a) and spring bed average (b) suspended solids concentration based on vertical profile data.

tenth percentile of the depth at every station on the cross-section. Plots of average concentration for each sub-group of data in every cross-section were arranged in sequence along the estuary to show the mean position and structure of the front in three dimensions. These plots show that the front is more prominent on spring than on neap tides and on ebb rather than flood tides. An average of all spring ebb data, Fig. 3, shows steeply inclined concentration contours across the gradient zone in the region between The Bridge and a line joining Barry and Burnham. Upstream from The Bridge, in the Bristol Deep, the front is duplicated by a similar, but smaller front on the Welsh side, whilst downstream from the Barry to Burnham line the concentration contours assume a more horizontal attitude.

Figure 3 illustrates the location and nature of the front in the study area but obscures the sequence of development and stability on a semi-diurnal time scale. In Fig. 4, the time series of cross-section plots represents individual traverses along standard line 1 during a spring tidal cycle of ~12.0 m range. The apparent slopes of the water surface are due to the rise and fall of the tide during the period taken by the vessel to steam along the line and have no dynamic significance. Figure 4 shows the effects of mixing of the sediment into the water column during accelerating phases of the tide and settling towards the sea bed on decelerating phases. Although well displayed at times when the tide is running in either direction, settling causes the front to be less evident at the surface and enhanced near the bed at slack water. However, the approximate position of the front remains constant on this time scale, although the concentration differences across it vary. Virtually the same sequence of changes was observed during time series at other ranges of tide at line 1 and also at the other standard lines.

Despite the averaging processes the position of the front remains well defined throughout spring tides (Figs 2, 3), most markedly at the bed during neap tides and during most of the semi-diurnal cycle (Fig. 4). These plan distributions and cross-sections suggest that, although the front is a marked feature on a regional scale, the gradient extends over 2 or more kilometres. In fact, from upstream of The Shoots to the Holm Islands the front can occasionally be seen at the sea surface in calm weather as a sharp interface separating 'blue' and 'brown' water. Continuous horizontal traverses made near the surface show that the front may be as little as 2-m wide at the upper end of the estuary (Fig. 5).

Unpublished data from IOS horizontal suspended solids traverses suggests that the front is not a passive feature associated with some controlling salinity and temperature structure

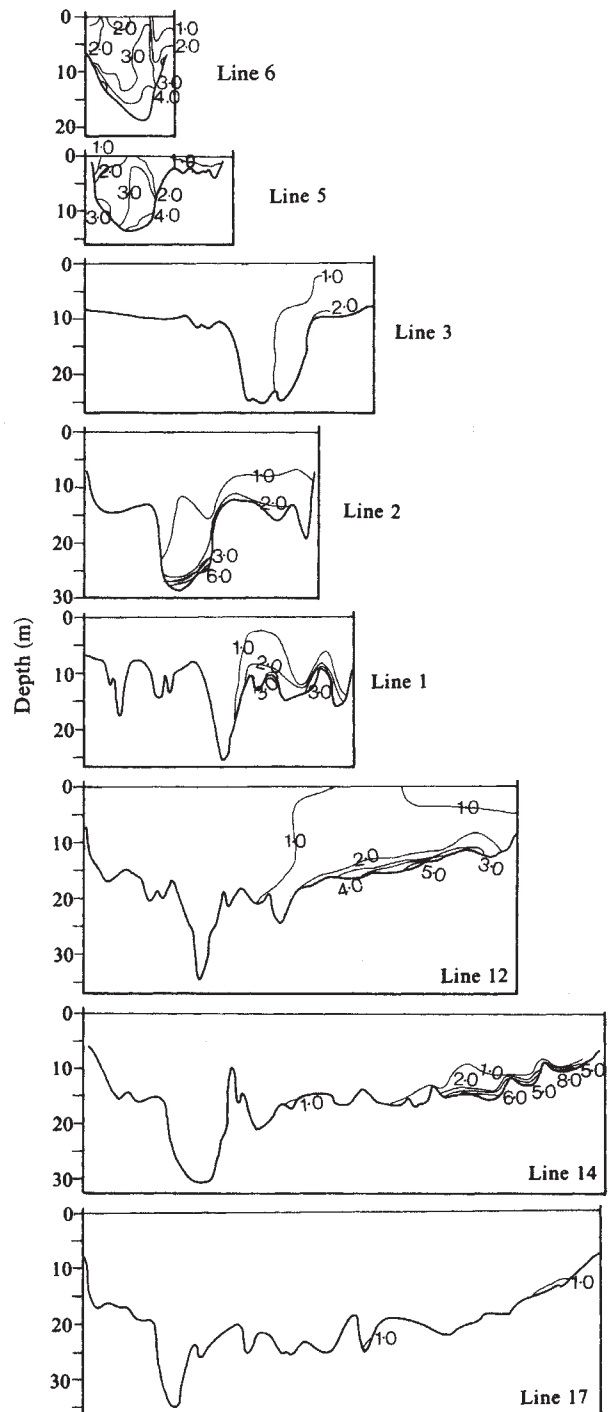


Fig. 3 Spring ebb average suspended solids distributions for cross-sections of the Severn Estuary and Inner Bristol Channel between Avonmouth and Watchet, concentration in g l^{-1} .

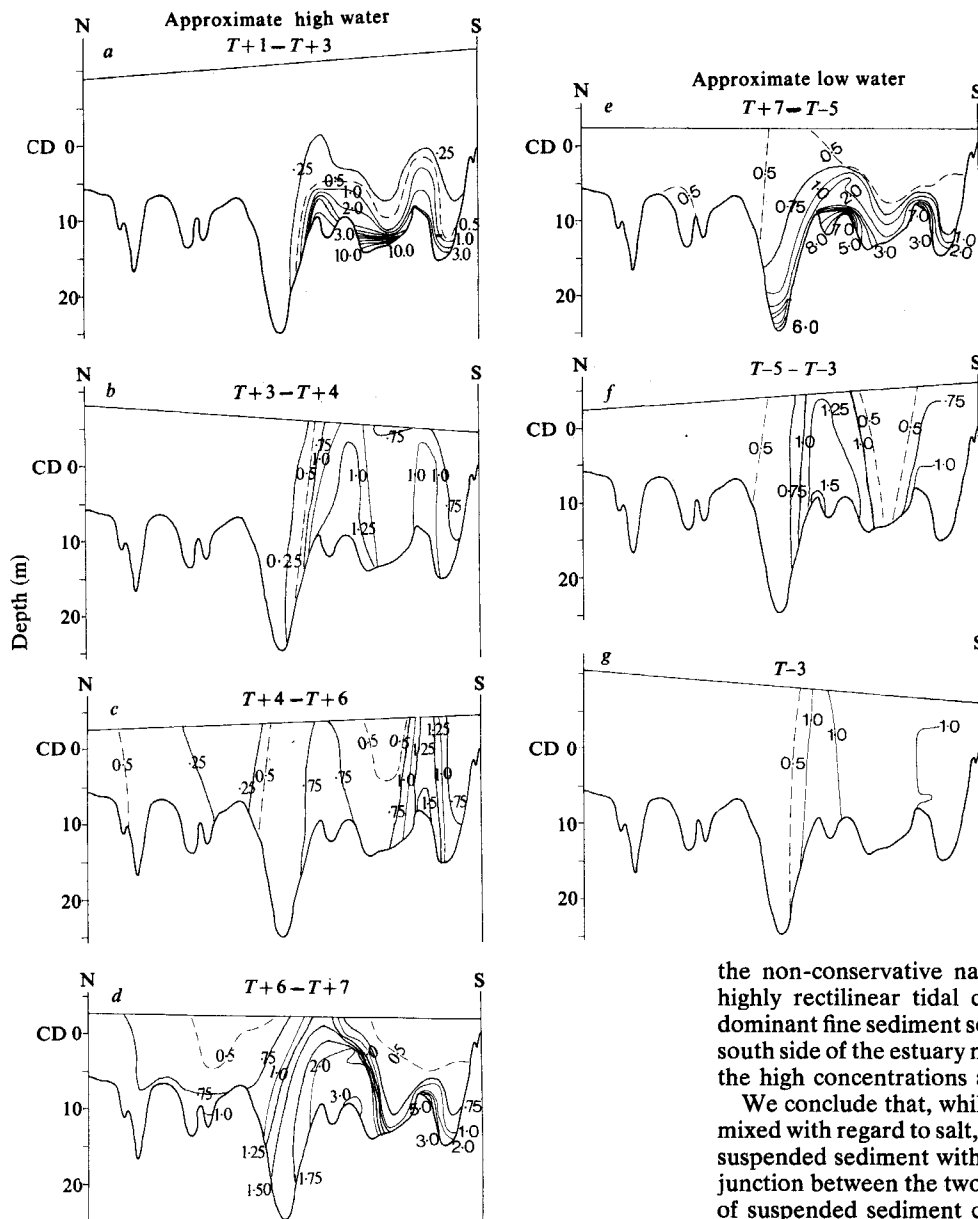


Fig. 4 Time series of cross-sections at Line 1 during a spring tide showing close association of front and main channel. Suspended sediment concentration in g l^{-1} . T numbers are GMT in hours + or - predicted HW at Avonmouth. CD, Chart Datum. a-d, Ebb tide sequence; e-g, flood tide sequence.

in the estuary. Published data show only low longitudinal and vertical salinity gradients. Strong lateral salinity gradients are confined to the coastal margins, even on neap tides, and no marked lateral gradients have been detected along the channel axis⁸. Neither is the phenomenon a plume-type front associated with the sediment discharge of a large river, since the estuarine suspended sediment concentrations are higher than in the inflowing rivers. Recent work (T. J. Smith, personal communication) has shown that the front is not associated with horizontal variations in vertical mixing rates and hence not analogous to the thermal or saline fronts frequently observed in British coastal waters¹⁰.

The front may be due to an interaction between the regional and local topography, Coriolis effects and lateral phase differences in tidal elevation across the estuary, which set up secondary water circulation cells in the plane of the cross-section, leading to convergence along the main channel axis. Velocity measurements over several tidal cycles made from an anchored vessel in the Holms Channel may be interpreted in support of this secondary circulation hypothesis. The vertical distribution of the lateral component of tidal mean velocity obtained from these measurements indicates a north-west going residual at the bed and a south-east going residual at the surface, in agreement with earlier data¹¹, placing the survey vessel on the Welsh side of a convergence. Such a circulation, combined with

the non-conservative nature of the suspended sediment, the highly rectilinear tidal current regime and the fact that the dominant fine sediment source area, Bridgwater Bay, lies on the south side of the estuary may be the main factors which maintain the high concentrations along the English side of the estuary.

We conclude that, whilst the Severn estuary is generally well mixed with regard to salt, it shows marked lateral partitioning of suspended sediment with a stably positioned front forming the junction between the two water masses. This lateral disposition of suspended sediment compliments the vertical stratification already reported in this estuary⁹. Evidently, whilst salt does get well mixed across the estuary the fine sediment does not, indicating that it is unwise to treat the dispersion of fine sediment in the same way as that of salt.

The residual water circulation in the estuary cannot be driven entirely by the saline density field because comparison of the

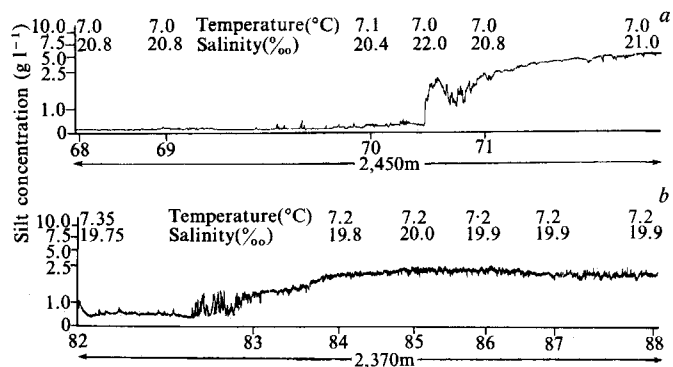


Fig. 5 Continuous horizontal traverses of suspended solids concentration with point readings of salinity and temperature across the front upstream of The Shoots. Towing depth 2.0 m. Concentration contrasts across the front a, $0.15\text{--}5.0 \text{ g l}^{-1}$; b, $0.4\text{--}2.2 \text{ g l}^{-1}$.

lateral and longitudinal density fields due to depth average salinity and depth average salinity plus suspended solids, shows that density differences due to suspended solids are at least equal to those due to salt¹². This comparison was achieved by calculating and comparing the depth mean density of the water column at individual stations across the estuary and along the estuary from Avonmouth to 3°30' W. Density differences computed using the actual suspended solids vertical profiles, as opposed to depth average values, show the suspended sediment density field to dominate.

As the suspended sediment load is strongly laterally partitioned and vertically stratified, sediment erosion and transport models should take account of the fact that any sediment entrained cannot be assumed to mix completely in lateral and vertical directions since the discontinuities represent a barrier to dispersion. Similarly, the presence of persistent lateral and vertical discontinuities within the suspended sediment concentration field also has an influence on the dispersion of those pollutants which travel with the sediment.

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Successive palaeomagnetic reversal records from Kauai

Scott W. Bogue & Robert S. Coe

Earth Sciences Board, University of California, Santa Cruz, California 95064, USA

Geomagnetic field reversal is a rapid phenomenon^{1,2}, perhaps taking only a few thousand years. Knowledge of how the field behaves during these brief and active periods may eventually help to constrain theories of the geodynamo and thus inform us about conditions in the Earth's core. Unfortunately, reliable and detailed palaeomagnetic records of transitional field behaviour are few, almost entirely from Northern Hemisphere sites, and heavily biased toward reversals of the reverse-normal (R–N) sense. We report here new data from basalts on the island of Kauai, Hawaii, which have recorded successive R–N and N–R field reversals. The sequences of changing field directions for the two reversals are very similar, as would be expected if a standing nondipole component controlled the transitional field. The data are also consistent with zonal flooding models in which the reversal process depends on the sign of the field. Other reversal records, however, cannot be explained by these same models unless the standing field or flooding process changes between some reversals.

Two simple models of the reversing field have been proposed in the past 5 years^{3,4}. The two involve conceptually different source mechanisms and, in their purest forms, make distinct predictions of transitional field behaviour. Although it is possible that no single process controls transitional fields, we will treat these two models as alternative explanations of palaeomagnetic records of field reversals.

Hoffman's model^{3,5} of the reversing field is based on the notion that the reversal process 'floods' through the fluid core

from a localized zone or point of initiation. The entire dipole source remains active, but normal and reverse flux is produced simultaneously in different regions. The resulting transitional field depends on how the reversal process propagates through the dipole source. If the reversal initiates at either of the poles or the equatorial zone of the core, essentially axial quadrupolar or octupolar fields, respectively, will predominate during the middle stages of the reversal process. In these purely axisymmetric cases, reversal of the field vector at the Earth's surface occurs by rotation in the north–south vertical plane; the equivalent virtual geomagnetic pole (VGP) moves along the great circle defined by the geographical poles and the observer's site. Depending on the sense of the reversal, where in the core source region the reversal process initiates, and the hemisphere of the observer, the VGP path for the reversal will be 'near sided' (same longitude as the site) or 'far sided' (180° away)⁶.

In contrast to the model of Hoffman, which we will refer to as the flooding model to emphasize its active character, is the standing field model⁴. The latter is a special case of the long held idea⁷ that, during reversals, the main dipole field diminishes greatly while at least parts of the nondipole field do not. In particular, the standing field model requires a stationary component of the nondipole field to persist unchanged through dipole reversals. Palaeomagnetic evidence suggests that the dominant part of the nondipole field changes sign with the dipole field^{8,9}. Thus, the postulated standing field would have to be in some sense independent of both the dipole and the rest of the nondipole fields.

In the standing field model, the transitional field at any site is the sum of a steady nondipole component plus an axial dipole component that changes only in magnitude and sign. As with simple cases of the flooding model, the equivalent VGP paths are longitudinal great circles. There is, however, no site dependence of path longitude unless the standing nondipole field direction everywhere lies in the north–south vertical plane.

Hoffman¹⁰ has recently proposed a test to distinguish between the flooding and standing field models. The former model predicts that, as long as a reversal is initiated at the same zone in the core each time, successive reversals will be characterized by transitional VGP paths that differ in longitude by 180°. The latter predicts identical VGP paths for successive reversals. Records of back-to-back reversals thus directly test the two models.

On the island of Kauai, Hawaii (22° N), we have collected samples through R–N and N–R transition zones. The thin, shield-building basalt flows of the Napali Formation (Fig. 1), which record the R–N reversal, have yielded K–Ar ages ranging from 4.5 to 5.6 Myr (ref. 11). The thicker, caldera-filling flows of the Olokele Formation (Fig. 1) have not been dated, but can be shown on geological grounds¹² to be synchronous with the upper portion of the Napali sequence. The transition zones in the Napali and Olokele Formations, however, are at nearly the same elevation. In addition, extensive reconnaissance with a portable fluxgate magnetometer has shown that all Napali flows exposed above the R–N horizon and all Olokele flows below the N–R horizon are normally magnetized. It is thus very likely that the two transition zones record a back-to-back, R–N–R reversal pair from the early Pliocene.

Equal-area plots of the flow-averaged palaeomagnetic directions from the two transition zones are shown in Fig. 2. Seven samples per flow were collected, and alternating fields of 100–200 Oe reduced substantially any secondary components of magnetization that were present. We sampled the R–N transition at three widely separated localities (Fig. 1), and the sequence shown in Fig. 2a is based on the simplest correlation of directions from the three sections. We sampled only a single section of the N–R transition because of the thick, horizontal character of the caldera basalts.

Neither transition zone displays an abundance of intermediate field directions. This lack of detail may be interpreted as the result of ill-timed lulls in volcanic activity both times the field was reversing. An alternative hypothesis is that the duration of

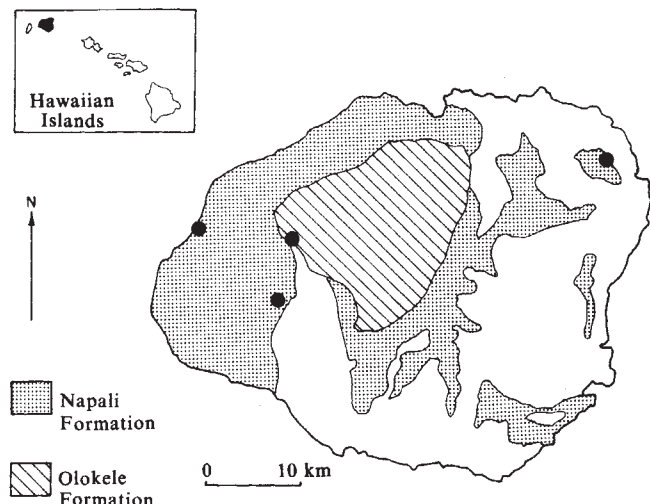


Fig. 1 Map showing outcrop extent of Napali and Olokele Formations on Kauai. Dots indicate locations of palaeomagnetic sampling sites.

intermediate field directions was very short. Our observation that contacts between flows were almost never marked by weathered zones or soil horizons supports the latter idea. In addition, the palaeomagnetic results show clearly that the three sections across the Napali reversal horizon are not identical, and thus that lavas on the flanks of the ancient Kauai volcano were of rather local extent. This pattern is entirely consistent with the modern eruptive style on the big island of Hawaii. Including the Olokele site, therefore, there are a total of four widely separated and essentially independent transitional sections that show this paucity of distinct intermediate directions. These two lines of evidence are mutually consistent, and we tentatively infer that the major change in direction occurred extremely rapidly during both reversals.

In spite of the lack of detail, and regardless of its cause, the pattern of change in field directions is clear for both transitions and is sufficient to apply Hoffman's¹⁰ test. In the R-N transition, the field direction remains southerly while shallowing to nearly horizontal. A single layer with normal polarity occurs among these lower flows. We have examined the anomalous unit and are convinced that it is a flow and not a sill; examples of the latter are easily recognized on Kauai by their distinctive jointing and much greater resistance to weathering. Thus the ancient field apparently underwent a rapid 'flip' in direction during the transition much like those observed in several other detailed records¹³⁻¹⁵. The shallow southerly directions are followed by a steeply downward directed field vector which subsequently shallows to the expected normal field direction. In the N-R reversal, the same sequence is seen except in reverse; a steep northerly field direction precedes shallow southerly directions. Thus, both reversals are characterized by transitional directions that lie near the north-south vertical plane or, equivalently, by VGP paths that pass near the site.

At first glance, this site dependence is most naturally explained by the zonal flooding model. An awkward complication arises, however, because the R-N and N-R paths are so nearly identical. Their similarity requires the presumed flooding processes for the two reversals to have initiated in opposite regions of the core⁶. More specifically, if the transitional field was predominantly octupolar, as is consistent with the very low field intensities that have been inferred from other transition zones¹⁶⁻¹⁸, then flooding for the R-N reversal would have begun at the equator. For the N-R reversal, one is forced to conclude that the reversal somehow initiated simultaneously at both poles. Similarly, quadrupolar transitional fields require initiation at the south pole for R-N reversals and at the north pole for N-R reversals.

If generally true, systematic behaviour such as just described would mean that the postulated flooding mechanism, and hence

the reversal process, depends on the sign of the field. Global palaeomagnetic data suggest that statistically significant and long-term differences do exist between the morphology and stability of normal and reversed geomagnetic fields¹⁹, and thus that asymmetry in the reversal process might be allowed. Such asymmetry, however, is intuitively unappealing and cannot arise directly from the basic dynamo equations^{6,19}.

Theoretical considerations aside, it is important to ask whether the regularities predicted by the flooding models are discernible in the rest of the palaeomagnetic record. Near-sided VGP paths seem to predominate in the R-N reversal records presently available from Northern Hemisphere sites^{3,20}. This characteristic is evident in the R-N reversal from Kauai, supporting the general observation. A pattern in the N-R records, on the other hand, is not yet apparent. Several N-R transition zones from Iceland have been reported²¹⁻²³; shallow directions with easterly or westerly declinations are common, so that VGP paths are not strongly near or far sided. Shallow westerly directions are also found in a detailed N-R transition in volcanic rocks of the Shigarami Formation, Japan²⁴. Overall, however, this record shows the field tending towards far-sided behaviour. Two N-R records from California^{25,26}, which we will discuss in more detail with respect to the standing field model, are characterized by intermediate directions which are shallow and easterly. The equivalent VGP paths are slightly near sided. A pair of VGP paths from Russian sites^{27,28}, the only N-R data considered in detail in a recent review, are not longitudinally confined, but are generally far sided.

More convincing evidence of far-sided N-R behaviour has been found in back-to-back transition zones of Miocene age from Crete²⁹. The VGP path for the N-R reversal follows a longitudinal path almost exactly 180° from the site. This record and that from the Olokele Formation on Kauai represent our best examples of site-dependent N-R paths, yet the former is far sided and the latter near sided. Thus, the N-R data as a whole display no coherent trend and, moreover, the two most clearcut records conflict directly. This evidence suggests that, at least for the N-R transitions, the flooding mechanism varies considerably from one reversal to the next.

The similarity of the N-R and R-N reversal records from Kauai is easily explained by the standing field model. Reversals in the early Pliocene were quite frequent³⁰, and the Kauai data require a standing field that persisted for only a few hundred thousand years. Results from two California localities suggest that a standing field may persist for a much longer period.

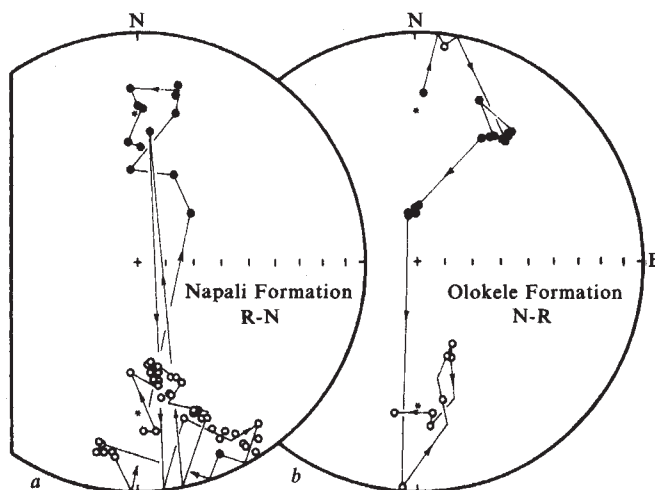


Fig. 2 a, Equal-area plot of flow-averaged directions from the R-N transition zone. Results from three sites in the Napali Formation are combined. ● (○) indicate downward (upward) directed magnetization vectors. Connecting lines and arrows show the stratigraphic order from oldest to youngest flows. Centre of plot marked by a plus sign, and ticks indicate 10° intervals. The axial dipole direction (asterisk) is shown for comparison. b, Directions from the N-R transition zone in the Olokele Formation.

Volcanic rocks from Clear Lake, California²⁵, record intermediate directions from two N-R reversals that occurred several hundred thousand years before the Matuyama-Brunhes transition. The VGP's equivalent to these directions fall right on the Matuyama-Brunhes R-N transition path that Hillhouse and Cox⁴ found at Lake Tecopa, also in California. Even more remarkable is a detailed record of the Gauss-Matuyama transition in dry lake sediments from Searles Valley, California²⁶. This reversal, 1.6 Myr earlier still and N-R, also repeats the Lake Tecopa path. Note that these three VGP paths are not strongly near or far sided, the VGPs passing almost 90° to the east of the site. Such grossly nonaxisymmetric field behaviour, nearly identical for reversals of both senses, can arise only in very special cases of the generalized flooding model⁵. Thus, the California results more strongly support the standing field model than do the Kauai data. More significantly, the ages of the three sites in California imply that the standing field, if that is what controlled the transitional fields, remained unchanged for several millions of years.

The back-to-back reversal records from Crete²⁹ again provide conflicting evidence. The VGP paths from these two reversals differ from one another by 135°. Marine sediments such as those now exposed on Crete may not record changing field directions as faithfully as rapidly deposited lake sediments or volcanic rocks. Nevertheless, it is difficult to dismiss the general features of these reversal records, especially the significant longitudinal separation of the VGP paths. Unless the standing field can occasionally change between reversals or be masked by other effects (flooding processes?), the results from Crete cannot be reconciled with the standing field model.

An additional problem is that the concept of standing fields has no clear independent basis. Yukutake and Tachinaka³¹ mathematically divided the historic nondipole field into standing and drifting parts. The analysis provided a better description of secular variation, but the separation of the field into two parts is arbitrary; it allows but is not strong evidence for an actual standing field. Moreover, analysis of historical field behaviour is not sufficient to demonstrate that a field component can last for millions of years as required by the model; such evidence must come from palaeomagnetism. The 'standing fields' that emerge from analyses of global palaeomagnetic data¹⁹ are by definition statistical; that is, they represent long-term properties of the time-averaged geomagnetic field. Standing fields of the sort needed for the reversal model are again allowed, but in no way proven, by analysis of the time-averaged field. In short, no standing nondipole field capable of controlling transitional fields has been observed, palaeomagnetically or otherwise, except by inference in some of the transition zone data.

In conclusion, the similarity of VGP paths in back-to-back reversal records from Kauai and in several records from California favours the standing field model, according to the test proposed by Hoffman¹⁰. Conversely, the large latitudinal separation of VGP paths in back-to-back reversal records from Crete and the predominance of near-sided VGP paths in the available R-N records from the Northern Hemisphere favour the zonal flooding model. However, unless the standing field or flooding process can change, at least occasionally, from reversal to reversal, neither model can explain all of the available transition data. It may thus be too simple to assume that a single type of transitional field is always present during reversals. Furthermore, palaeointensity data we are currently analysing for the Kauai R-N reversal suggests that more complex versions of these mechanisms may be required to model even a single reversal. These results will be discussed elsewhere.

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Rapid growth of a deep-sea manganese nodule

J. L. Reyss*, V. Marchig† & T. L. Ku‡

* Centre des Faibles Radioactivités, Laboratoire mixte CNRS-CEA, Avenue de la Terrasse, 91190 Gif-sur-Yvette, France

† Bundesanstalt für Geowissenschaft und Rohstoffe, Hannover, FRG

‡ University of Southern California, Los Angeles, California 90007, USA

Recent studies on marine ferromanganese nodules have suggested a relationship between their growth rate and their chemical composition¹⁻³. The suggestion is based on the hypothesis that the metals in nodules have two sources of supply: bottom seawater (hydrogenous) and sediment pore water (diagenetic)⁴⁻⁷. The diagenetic source calls for upward diffusion of Mn mobilized in a deeper, low *Eh* zone of the sediment column and its subsequent precipitation in an oxidized surface zone⁸. Addition of the remobilized Mn to surface nodules would augment their accretion rate. It would also render the oxide layers to contain higher Mn/Fe ratios than layers resulting from hydrogenous precipitation. We report here a radiometrically determined accretion rate of 168 ± 24 mm Myr⁻¹ for a deep-sea manganese nodule from the Peru Basin in the Pacific. This rate is about two orders of magnitude higher than that frequently found in nodules of deep-sea origin, and emphasizes the importance of sediment diagenetic processes on the growth of manganese concretions.

When compared with their open-ocean counterparts, manganese deposits from shallow-water, continental margin environments commonly show growth rates that are three or more orders of magnitude faster ($1-10^3$ mm kyr⁻¹ compared with $1-10$ mm Myr⁻¹), and Mn/Fe ratios that are generally higher, although with large variations (0.5-50 compared with ≈ 1) (refs 5-9). Aside from the distance involved in the continental runoff supply of metals, such contrasts have generally been held to reflect the preponderance of the aforementioned one source of Mn over the other. That is, nearshore nodules are principally of diagenetic origin whereas pelagic nodules are hydrogenous^{5,10-12}. However, we intend to assert here that the above is not meant to be a dichotomy between nodule growth in shallow marine and in deep abyssal sedimentation regimes. Although the *Eh*-controlled diagenetic reactions should depend geographically on surface productivity and sedimentation rate, nodules of deep-ocean occurrence may

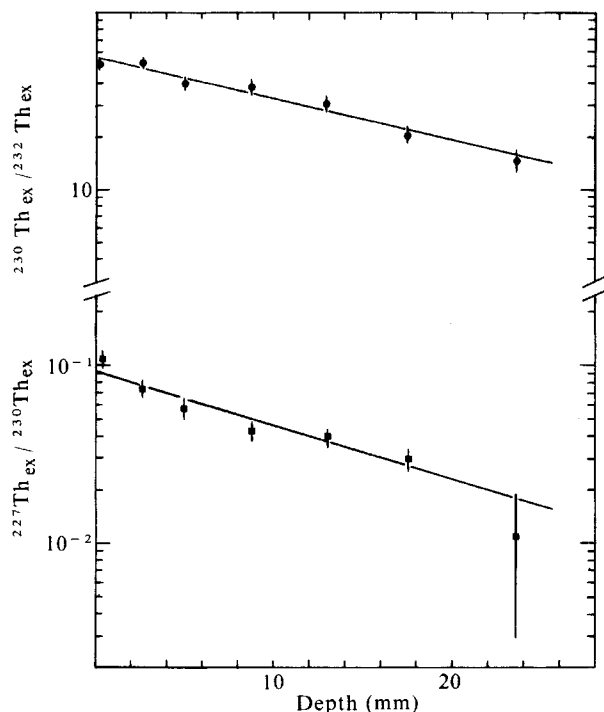


Fig. 1 Plots of $^{230}\text{Th}/^{232}\text{Th}$ and ^{227}Th (as ^{231}Pa)/ ^{230}Th activity ratios as a function of depth in nodule 278 DK. The mean accretion rates (●, $V = 173 \pm 25 \text{ mm Myr}^{-1}$, ■, $V = 162 \pm 40 \text{ mm Myr}^{-1}$) shown are determined from the weighted least-square fits (solid lines). Half lives used are 75,200 yr and 34,300 yr for ^{230}Th and ^{231}Pa , respectively.

themselves exhibit, as manifestations of regional variations in diagenetic influence, a range of Mn/Fe and accretion rate values.

As Halbach and his co-workers pointed out^{7,13}, there is a south-to-north increasing trend in the Mn/Fe ratio of nodules in the Peru Basin, south-east Pacific (the 'Sonne Basin') suggestive of a rising influence of diagenetic supply of Mn towards the north, which lies near the equatorial region of high surface biological productivity. Halbach *et al.*⁷ estimated that nodules in this northern region grow at a rate of $>60 \text{ mm Myr}^{-1}$, as opposed to rates of a few mm Myr^{-1} in the south. However, these estimates are very uncertain as they are based on extrapolation of the crude relationship between Mn/Fe and growth rate obtained by Heye and Marchig².

We have determined the accretion rate of a nodule from the northern part of the basin using U and Th series isotopes. The specimen (sample 278 DK) is a large, spheroidal nodule ~15 cm in diameter. It was retrieved from 4,240 m water depth at $7^{\circ}40.0' \text{ S}$ and $91^{\circ}59.5' \text{ W}$ during cruise SO 11/1 of the german RV Sonne. Its high Mn/Fe ratio ≈ 60 (Table 1) and its botryoidal, highly-mammillated constitution conform with the Halbach^{13,14} Ac type nodule, the formation of which they describe

as being largely influenced by sediment diagenesis near the sea floor. Each mammillae consists of alternate compact and porous laminations ranging in thickness from 1 to 7 mm.

We have measured U and Th series isotopes in nine oxide layers from surface to 25.5 mm inward, and in sediments attached to the nodule surface. After total acid dissolution of the samples, we measured ^{238}U , ^{234}U , ^{232}Th and ^{230}Th using procedures similar to those of Ku and Broecker¹⁵. The isotope ^{231}Pa was measured through its granddaughter ^{227}Th , assuming the two are in secular equilibrium. Results are presented in Table 1.

The isotopes found in nodule layers have three possible sources: seawater, pore water and detrital sediment included in the oxides. (No active volcanic activity exists in this part of the ocean floor; thus direct precipitation from hydrothermal solution as a source can be discounted.) From their Al-Si contents (Al $< 0.8\%$, Si $< 3\%$), the oxide layers seem to include $< 10\%$ by weight of detrital sediment.

The nodule material contains two to three times more U than the associated sediment, indicating that most of their U isotopes come from seawater and/or pore water. Data on the $^{234}\text{U}/^{238}\text{U}$ ratio (Table 1) are consistent with this assessment. This ratio in seawater, pore water and deep-sea sediment has been reported as, respectively, 1.14, 1.17–1.23, and < 1.00 (refs 16–19). For the other isotopes: ^{232}Th , ^{230}Th , and ^{231}Pa (^{227}Th), all three sources may have contributed to their presence in the oxide layers. Unlike U, evidence for the possible contribution of the Th isotopes from detrital sediment source includes that ^{232}Th in the sediment is about an order of magnitude higher than in the oxides, and that $^{230}\text{Th}/^{232}\text{Th}$ ratios in the surface layers and in the sediment are similar (Table 2). Although little is known about the Th and Pa isotopes in pore fluid, Moore *et al.*²⁰ have shown the supply of ^{231}Pa and ^{230}Th from this source to the bottom face of a nodule. According to them, a preferential loss of ^{231}Pa relative to ^{230}Th from the sediment to its pore fluid may occur. The $^{227}\text{Th}/^{230}\text{Th}$ ratio of 0.11 measured for the outermost layer of nodule 278 DK as compared with the ratio of 0.07 for the associated sediment (Table 2) accords with this possibility.

The accretion rate of 278 DK can be estimated from the rate of decrease of excess ^{230}Th ($^{230}\text{Th}_{\text{ex}}$) $T_{1/2} = 75.2 \text{ kyr}$ and/or ^{231}Pa ($^{231}\text{Pa}_{\text{ex}}$) $t_{1/2} = 34.3 \text{ kyr}$ in the nodule. The use of $^{230}\text{Th}_{\text{ex}}/^{232}\text{Th}$ and $^{231}\text{Pa}_{\text{ex}}/^{230}\text{Th}_{\text{ex}}$ activity ratios is preferred to the use of specific activities (for example, d.p.m. $^{230}\text{Th}_{\text{ex}}$ per gramme of nodule material), in view of the nodule's textural variations. Indeed, the considerable variation of the ^{230}Th specific activities with depth (Table 1) is largely removed when normalized against those of ^{232}Th and ^{227}Th (^{231}Pa) (Table 2).

The sedimentary source for U is unimportant and the uranium precipitated from seawater or pore water is assumed to carry little or no ^{230}Th and ^{231}Pa . Thus the U-supported ^{230}Th and ^{231}Pa should be nil when deposited and increase towards secular equilibrium with uranium at depth. The rate of increase of these U-supported ^{230}Th and ^{231}Pa , as well as the rate of decrease of $^{230}\text{Th}_{\text{ex}}$ and $^{231}\text{Pa}_{\text{ex}}$ mentioned above, depends on the accretion rate and the decay constants involved. Using an iterative procedure analogous to that of Koide *et al.*²¹ for the sediment ^{226}Ra geochronology, we calculate the $^{230}\text{Th}_{\text{ex}}/^{232}\text{Th}$ and

Table 1 Chemical and radiochemical data for nodule 278 DK and associated sediment

Depth interval (mm)	^{234}U (d.p.m. g^{-1})	$^{234}\text{U}/^{238}\text{U}$	^{232}Th (d.p.m. g^{-1})	^{230}Th (d.p.m. g^{-1})	^{227}Th (d.p.m. g^{-1})	Fe* (%)	Mn* (%)
0–0.5	3.32 ± 0.16	1.09 ± 0.05	0.280 ± 0.017	14.5 ± 0.7	1.60 ± 0.13	0.58	41.1
1.3–4.0	4.35 ± 0.21	1.14 ± 0.06	0.240 ± 0.012	12.9 ± 0.6	0.97 ± 0.08	0.72	41.6
4.9–5.0	4.35 ± 0.25	1.08 ± 0.06	0.350 ± 0.024	15.0 ± 0.9	0.90 ± 0.09	0.64	45.0
5.0–12.5	4.97 ± 0.24	1.08 ± 0.05	0.427 ± 0.021	18.0 ± 0.9	0.84 ± 0.06	0.56	55.2
12.5–13.5	2.90 ± 0.13	1.17 ± 0.06	0.700 ± 0.042	23.1 ± 1.3	0.97 ± 0.08	1.58	39.1
15.5–19.5	2.87 ± 0.14	1.12 ± 0.06	0.384 ± 0.022	9.8 ± 0.5	0.35 ± 0.03	0.64	42.4
21.5–25.5	3.32 ± 0.16	1.12 ± 0.06	0.190 ± 0.013	5.2 ± 0.3	0.16 ± 0.02	0.72	36.2
Sediment	1.27 ± 0.08	1.04 ± 0.06	2.01 ± 0.12	105 ± 6	7.0 ± 0.6		

Errors quoted are based on counting statistics.

* Atomic absorption.

Table 2 $^{230}\text{Th}_{\text{ex}}/^{232}\text{Th}$, $^{227}\text{Th}_{\text{ex}}$ (as $^{231}\text{Pa}_{\text{ex}}/^{232}\text{Th}$, and $^{227}\text{Th}_{\text{ex}}$ (as $^{231}\text{Pa}_{\text{ex}}/^{230}\text{Th}_{\text{ex}}$ ratios

Depth interval (mm)	$^{230}\text{Th}_{\text{ex}}/^{232}\text{Th}$	$^{227}\text{Th}_{\text{ex}}/^{232}\text{Th}_{\text{ex}}$	$^{227}\text{Th}_{\text{ex}}/^{230}\text{Th}_{\text{ex}}$
0–0.5	51.6 ± 3.1	5.69 ± 0.47	0.110 ± 0.011
1.3–4.0	51.4 ± 2.7	3.84 ± 0.35	0.075 ± 0.008
4.9–5.0	40.0 ± 2.9	2.33 ± 0.28	0.058 ± 0.008
5.0–12.5	37.8 ± 2.1	1.64 ± 0.15	0.043 ± 0.005
12.5–13.5	30.9 ± 2.0	1.25 ± 0.12	0.040 ± 0.005
15.5–19.5	21.0 ± 1.5	0.64 ± 0.08	0.030 ± 0.004
21.5–25.5	14.9 ± 2.2	0.16 ± 0.12	0.011 ± 0.008
Sediment	51.6 ± 3.2	3.45 ± 0.32	0.067 ± 0.009

Ratios were calculated using an iterative procedure analogous to that of Koide *et al.*²¹ for nodule 278 DK and associated sediment.

$^{227}\text{Th}_{\text{ex}}/^{230}\text{Th}_{\text{ex}}$ values corresponding to their respective least-squares fits for an exponentially decreasing function with depth. These values are listed in Table 2 and plotted in Fig. 1. The slopes of the fits give accretion rates estimated for the nodule: $173 \pm 25 \text{ mm Myr}^{-1}$ from the $^{230}\text{Th}_{\text{ex}}/^{232}\text{Th}$ plots and $162 \pm 40 \text{ mm Myr}^{-1}$ from the $^{227}\text{Th}_{\text{ex}}/^{230}\text{Th}_{\text{ex}}$ plots.

Such rapid accumulation rates have been radiometrically determined for the ridge-hydrothermal and shallow-water manganese deposits^{9,22–24}. This is the first determination for a manganese nodule of deep-water origin. Our results support the concept that diagenetic remobilization of Mn in sediment column could significantly affect the growth of manganese nodules at the sediment–water interface^{3,7}.

Preliminary results on a nodule from the southern part of the basin show a slow growth rate of 4.5 mm Myr^{-1} and a ratio Mn/Fe of about 3. The trend is that nodules largely have a diagenetic origin (278 DK type) in the north and are hydrogenous in the south as deduced from their Mn/Fe ratio⁷, the two processes may occur concurrently in intermediate areas. From these data we could expect slow precipitation from bottom sea water, plus, depending on the ambient oxidation potential, or thickness of the surface oxic layer of the sediment, a more or less important diagenetic contribution, resulting in variable growth rates ranging from millimetres to hundred of millimetres per million years. Work in progress should elucidate the relationships between the growth rate and composition of nodules and the oxidative characteristics of their sediment substrates in the Peru Basin.

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Biogenic calcite structures forming in Lake Fryxell, Antarctica

Robert A. Wharton Jr*, Bruce C. Parker*, George M. Simmons Jr*, Kenneth G. Seaburg* & F. Gordon Love†

* Department of Biology and † Department of Geological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA

Stromatolites are organosedimentary structures produced by microorganisms which have been used extensively in the interpretation of Precambrian and, to a lesser extent, Phanerozoic history^{1–5}. Modern stromatolites are generally restricted to hot springs or warm, hypersaline and/or alkaline aquatic environments³. Recently, however, we documented⁶ the presence of modern stromatolites in several cold, perennially ice-covered Antarctic dry valley lakes. Here, we report a finding in Lake Fryxell, Antarctica, of biogenic calcite structures not observed in our previous study of other Antarctic lakes. These structures were laminated, indurate, unbranched, and had either a vertical or horizontal orientation. We infer that these calcite structures are biogenic in origin, and hence stromatolitic based on the following evidence: (1) the presence of an actively metabolizing algal mat on their surface; and (2) an internal composition of filamentous blue-green algal cell wall fragments, diatom frustules and calcite crystals.

Lake Fryxell (77°37' S, 163°07' E) is located at the eastern end of Taylor Valley in southern Victoria Land, Antarctica (see Table 1 for physicochemical features). It is 16 m above sea level, 20 m deep, 5.2 km long, 1.7 km wide and has a surface area of 5.4 km². This latitude receives approximately 4 months each of sunlight, twilight and darkness. The perennial 4–5-m thick ice-cover of Lake Fryxell overlies ~15 m of water <4°C and allows penetration of <2% of the incident photosynthetically

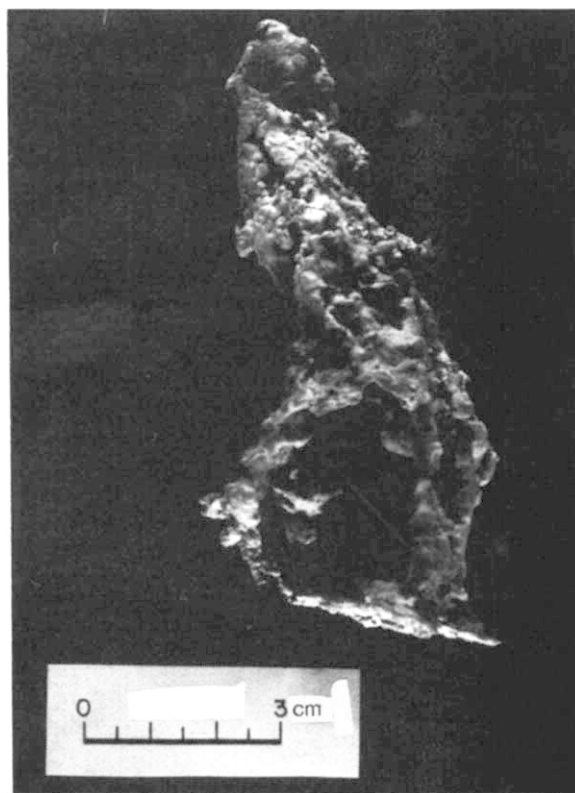


Fig. 1 Typical vertical calcite (determined by X-ray diffraction) structure collected from beneath 0.2–1.0 cm of actively metabolizing algal mat at a depth of ~7 m, Lake Fryxell, Antarctica.

Table 1 Selection of physical and chemical variables of Lake Fryxell on 28 December 1980

Depth (m)	Temperature (°C)	% Transmission*	O ₂ (mg l ⁻¹)	CO ₂ (mg l ⁻¹)	Alkalinity (mequiv. l ⁻¹)	Ca (mg l ⁻¹)	Mg (mg l ⁻¹)	pH	Specific conductance (µS cm ⁻¹)
5	1.1	1.41	23.5	14.1	6.74	29	17	7.5	1,200
6	2.0	1.31				21, 77	40, 129		1,370
7	2.8	0.81	37.0	19.4				7.7	2,520
8	3.2	0.62	10.7	66.0		31	124	7.3	3,320
8.5	3.6	0.46	4.1	91.5	19.52			7.3	3,720
8.75	3.7	0.41	2.3	88.9				7.4	3,930
9	3.8	0.30		86.2	25.84			7.4	4,280
10	3.8	0.08	0	135.5		35	184	7.4	4,300
11	3.8	0.02							5,200
12	3.7	0.01	0	207.7		31, 33	284, 229	7.4	5,800
13	3.7	0.005							6,300
14	3.7	0.003							6,900
15	3.4	0	0	240.2		43	311	7.3	7,200
16	3.2	0				27	331		7,400
17	3.0	0							7,700
18	2.8	0							7,900

Calcium and magnesium data are from ref. 16, first no. and ref. 17, second no.

* Per cent transmission of surface photosynthetically active radiation (400–700 nm wavelength light) through ice and water at depth indicated.

active radiation (400–700 nm wavelength) during the austral summer. The ice cover also prevents free exchange of gases with the atmosphere, wind-generated turbulence and internal mixing of the lake water. This is exemplified by oxygen supersaturation in the upper few metres of the water column, descending to anaerobic conditions below 10 m. The lake lacks outflowing streams, and receives sediment, salts and nutrients from glacial meltstreams during the austral summer.

During the 1980–81 austral summer we melted two dive holes through the ice-cover of Lake Fryxell and SCUBA divers observed and photographed the sub-ice environment. Benthic cores collected at a water depth of 7–9 m revealed that the upper mat layer (0.2–1.0 cm thick) contained actively metabolizing organisms. Metabolic activity was inferred from microscopic examination of motile blue-green algal filaments and diatoms in the mat, isolation of viable microorganisms, and from measurements of photosynthetic pigments, ATP and ¹⁴C primary productivity. This top layer of mat consisted primarily of the blue-green alga *Phormidium frigidum* Fritsch, pennate diatoms (for example, *Navicula* sp., *Caloneis* sp.) and heterotrophic bacteria. Below this layer were alternating bands of organic-rich and sediment-rich layers to a depth of ~50 cm. Five organic layers, isolated from 7–32 cm in the sediment, had an average organic matter content of 8.7%. These organic and sediment laminations are similar to the Antarctic stromatolites previously reported by Parker *et al.*⁶. Embedded within these sediments were discrete structures composed of calcite (Fig. 1).



Fig. 2 Photograph of the benthos at a depth of ~7 m showing surface morphology of living algal mat in which trapped photosynthetically produced oxygen has induced lift-off from the substrate (maximum vertical relief, ~10 cm).

These calcite structures were laminated, indurate and unbranched and had developed either a vertical or horizontal orientation. The vertical structures (1–5 cm in diameter, 1–10 cm long) were usually hollow, terete and covered by a 0.2–1.0-cm thick layer of algal mat (Fig. 2). At a depth of ≥ 50 cm in the sediment was a stratiform layer of calcite ~5 mm thick. Microscopic examination of these structures revealed diatom frustules and filamentous blue-green algal cell wall fragments ~1–2 µm diameter, the latter resembling filaments of *Phormidium*.

The vertical calcite structures in Lake Fryxell apparently result from the growth of the algal mat in dimly-lit, shallow, non-turbulent water. Austral summer photosynthetic production of O₂ causes gas bubbles to be trapped within the sticky mucilaginous algal mat matrix and initiates a vertical growth habit^{6–10}. In other Antarctic lakes observed, buoyant forces sometimes tear the mat matrix, and the algal mat detaches completely from the substrate^{6–13}. However, in Lake Fryxell, much of the gas-charged mat is stabilized by the precipitated calcite and does not tear loose from the substrate. The hollow nature of the structures suggests that calcite precipitation follows the orientation of the growing mat.

The vertical structures in Lake Fryxell closely resemble some of the calcite-impregnated defluidization structures recently reported from Mono Lake, California¹⁴. However, in contrast to the Mono Lake structures, which were considered to be formed by physicochemical carbonate deposition, the Antarctic structures seem to be primarily the result of algal activity. The horizontal structures are formed either at greater depth in the sediment where no light is available for photosynthesis or at depths where photosynthetic activity is insufficient to accumulate trapped oxygen, which results in a flat mat growth habit.

Several unique biological, physical and chemical features of Lake Fryxell produce and preserve these stromatolitic structures⁶. (1) While a few tardigrades, rotifers and nematodes inhabit the algal mats in the shallow peripheral margins of the lake, major burrowing and browsing organisms (such as cladocerans, copepods, annelids, gastropods, insect larvae and fish) are entirely absent¹⁵, leaving these mats essentially undisturbed by metazoans. (2) Sediment influx to the lake consists of periodic influxes of glacial meltstreams which flow at most 10–12 weeks of the austral summer, contributing insufficient sediment to obliterate the mats. (3) Except for localized mixing of lake water near glacial meltstream deltas, no internal mixing of lake water occurs which prevents disturbance of existing sediments. (4) Low temperatures and a paucity of metabolically active bacteria possibly slow the decomposition of organic matter. (5) Sufficient calcium and dissolved carbonate^{16,17} are available for the biogenic calcite precipitation¹⁸.

These Antarctic stromatolites may resemble ancient permanently submerged stromatolites of the Precambrian era^{6,7,9}. Because of low light intensities, lack of water turbulence and absence of burrowing and/or grazing metazoans, the benthos of Lake Fryxell may simulate some of the conditions that occurred in Precambrian deep subtidal marine or lacustrine environments which were dominated by stromatolite-forming microorganisms⁵. Thus, Lake Fryxell is an unusual habitat where modern stromatolites are forming, and further investigation may provide insight into the palaeoecology of Precambrian stromatolites.

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Neural reorganization during metamorphosis of the corpora pedunculata in *Drosophila melanogaster*

Gerhard Technau & Martin Heisenberg

Institut für Genetik und Mikrobiologie, 87 Würzburg, FRG

Adult fruit flies and *Drosophila* larvae have very different bodies and behaviour patterns. The question therefore arises: how much reorganization does the brain undergo during pupation? Some apparent shape changes have been documented, for example, the growth of the optic lobes and the separation of the prothoracic and suboesophageal ganglia^{1,2}. In the central brain, the issue is less clear. Most neurone cell bodies persist through metamorphosis³, and the general shape of certain neuropil regions is conserved. However an identified motoneurone in another holometabolous insect (*Manduca*) has been shown to change its dendritic arborization patterns during pupation⁴. Here we report that the mushroom body, a major neuropil area in the insect brain, is extensively reorganized during pupation (that is, many of the intrinsic fibres are broken down and made anew). This reorganization is cryptic—the overall morphology of the mushroom body is apparently constant throughout pupation. We have now studied this reorganization by examining two mutations (*mbd*, *mud*) which cause derangement of this structure during metamorphosis, and we report that this reorganization is one of the most extensive yet found in insect metamorphosis.

As in most arthropods, the mushroom bodies of *Drosophila* are two conspicuous substructures of the median protocerebrum arranged in mirror symmetry to the saggital midplane. The numerous, relatively small cell bodies of their intrinsic neurones (Kenyon cells) are situated in the posterior dorsal cortex. The calyx is formed by postsynaptic glomerulus-like arborizations of the Kenyon cell fibres and by collaterals of the antennal glomerular tract (AGT) as presynaptic elements. From there, the Kenyon cell axons grow as a large bundle—the peduncle—from back to front through the whole brain on either side of the central complex. Near the anterior margin of the neuropil, most of the axons divide, sending one branch towards the midplane (β -, γ -lobes), the other one upwards (α -lobe). Thus the whole stalk system consists of three arms (peduncle, α -lobe, β -, γ -lobes) oriented roughly parallel to the three main axes of the fly^{5–7}. It is this bizarre shape which makes the mushroom bodies already well discernible in the brain of the early first instar larva, where they are found in the same position and orientation as in the imago. Thus, at first sight, mushroom body development seems to imply no more than the addition of Kenyon cells and the elongation of their axons with the growth of the surrounding central brain neuropil.

Recently several mutants altering the morphology of the mushroom bodies have been described⁶. In two of them (*mushroom bodies deranged*^{KS65}, *mbd* and *mushroom body defect*^{KS63}, *mud*) the lobe and stalk system of the mushroom bodies is missing (not always completely in the case of *mbd*) and the calyces are enlarged. We noticed, to our surprise, that these mutant phenotypes occur only in the imago (Fig. 2). In the late third instar mutant larvae, the mushroom bodies have the same shape as in wild-type flies (Fig. 3). Twenty hours after puparium formation their neuropil is more or less completely degraded. Apparently, the newly growing 'imaginal' Kenyon cell axons fail to find their normal way through the central brain neuropil and remain at the site of the calyces, causing abnormal enlargement of these structures. Cell proliferation in the mushroom body cortex still seems to be normal in *mbd* (increased in *mud*). In these mutants, as in wild-type flies, neuroblasts are no longer detectable 3 days after puparium formation.

Replacement of larval Kenyon cell fibres is not a peculiarity of the mutants. A similar process occurs in wild-type individuals as well; apparently, only the course of newly growing 'imaginal' fibres is altered in the mutants. This reorganization of the wild-type mushroom body neuropil is documented by the following data.

Figure 1 shows for wild-type *Berlin* flies the number of Kenyon cell axons in the caudal peduncle, as a function of time measured from egg hatching. Counts were taken from electron micrographs of cross-sections through the peduncle, a few micrometres anterior to the calyx. The number of axons increases from about 300 in the first instar larva to about 2,100 in the late third instar larva. At the time of puparium formation, the number of axons drops abruptly. Already 12 h later, it is reduced by about 40%. From then on, it increases again until it reaches the imaginal level (2,200 fibres). From Golgi preparations of adult *Drosophila*, we know that Kenyon cell axons in the caudal peduncle have no ramifications, which could have contributed to the total cross-sectional count (K. F. Fischbach, personal communication). In electron micrographs of a series of longitudinal sections through the late larval peduncle, we found few ramifications (possibly belonging to extrinsic elements), implying that among the larval Kenyon cells as well, collaterals must be rare or even missing in this part of the mushroom bodies. Therefore we believe that the decrease of fibres in the peduncle of the early wild-type pupa reflects degeneration of whole larval axons rather than collaterals. The fact that Kenyon cell axons degenerate in the mutants supports this conclusion. Note that this is contrary to the situation of the motoneurone in *Manduca*⁴ where the axon is maintained.

We have no data on whether extrinsic elements are included in this process. However, we assume that, in the peduncle, these constitute only a small minority of the profiles. In the late larval

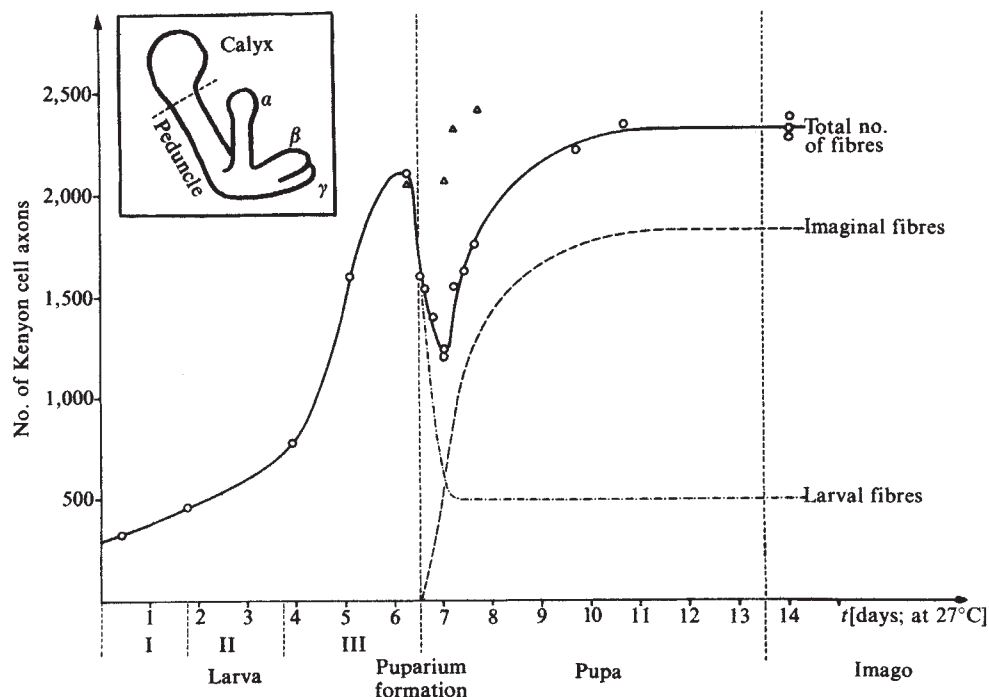


Fig. 1 Total number of Kenyon cell axons (including extrinsic elements) in the caudal peduncle of wild-type *Drosophila* (for plane of section see inset) as a function of developmental time starting at eclosion (zero time). Cell fibres are shown as open circles and cell bodies (relative counts) as triangles. Whereas the fibres of larval Kenyon cells degenerate within the first hours of pupal life, their cell bodies remain alive. The dash and dash-dot lines represent the hypothetical larval and imaginal fibre portions. The curves are based on the assumption that the bundle of (~500) thin fibres positioned centrally in the stalk system contains the only larval fibres which remain throughout metamorphosis.

stage the total number of profiles in the caudal peduncle is very close to that of Kenyon cell bodies. Hardly any tangential elements are observed which could be interpreted as penetrating extrinsic fibres or ramifications of them. Golgi preparations from adult flies rarely show extrinsic elements or specializations of intrinsic fibres in this part of the mushroom bodies. Finally, in the larva as well as the adult, cross-sections from different levels of the peduncle (except for the part close to the lobes) do not vary by more than 5–10% in their total number of profiles. Thus extrinsic elements appear to contribute little to the effect observed.

The cell bodies of the larval Kenyon cells remain alive: a staining procedure which clearly marks degenerated material (Mallory's solution of azur II and methylene blue applied to 1 μ m plastic sections) shows no sign of degeneration in the mushroom body cortex at any developmental stage in either wild type or the mutant *mbd*. This is confirmed by the fact that the number of Kenyon cell bodies does not decrease during the time of fibre reduction (Fig. 1). In the pupal stage, addition of new cells to the mushroom body cortex is much less than the number of new 'imaginal' fibres, implying that at least part of these fibres belong to regenerating Kenyon cells. Presumably, sprouting of new fibres continues while larval fibres are degenerating. In fact, scattered profiles of groups of extremely thin fibres, which may represent filipodia of growing axons are found throughout the degeneration period. Thus, loss of larval Kenyon cell axons

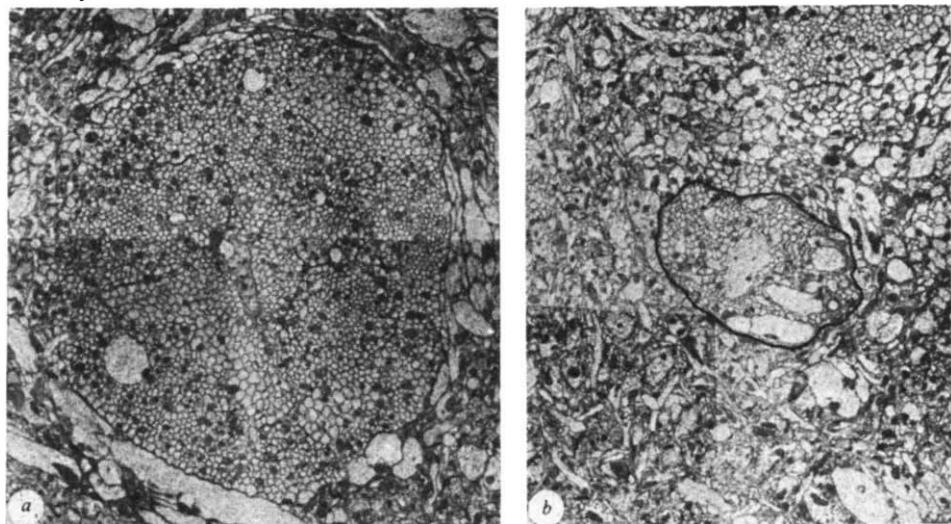
probably by far exceeds 40% (Fig. 1).

The degenerating neuropil shows typical histological alterations⁸ (Fig. 4) in all parts of the mushroom bodies. Axons have short, swollen segments containing electron-dense granules (presumably glycogen), lamellar bodies and an increased number of vesicular components (Fig. 4c). Ramifications of glial cells in this area are drastically enlarged. They are filled with osmiophilic material and axon fragments, which suggests their active role in the degeneration process (Fig. 4b). Twenty hours after puparium formation, the histological signs of degeneration have already disappeared.

The misrouting of the mutant Kenyon cell axons during metamorphosis is not yet understood. In larvae and early pupae of wild-type flies we find a bundle of very thin fibres located centrally in the peduncle and β -lobe which never shows any histological signs of degeneration (Figs 3a, 4a). In the mutant *mbd* this bundle is frequently missing. If the frequency of this larval defect correlates with the expressivity of the *mbd* phenotype in the adult, the role of this bundle may be seen as a guide for the ingrowing 'imaginal' fibres. (In the mutant *mud*, cross-sectional profiles in the larval peduncle are doubled in number and are small and uniform in diameter. No special bundle can be detected.)

Since experiments with the mutant *mbd* strongly suggest that the mushroom bodies participate in the evaluation of odors (M. Heisenberg, unpublished), it is tempting to speculate about the

Fig. 2 Cross-section through the peduncle of a, 7-day-old wild-type imago; b, an imago of the mutant *mushroom bodies deranged*^{KS65}. In flies of this mutant, the number of Kenyon cell fibres (here ~250) forming the peduncle is variable, while in most flies of the mutant *mushroom body defect*^{KS63} peduncles are not detectable. $\times 3,599$.



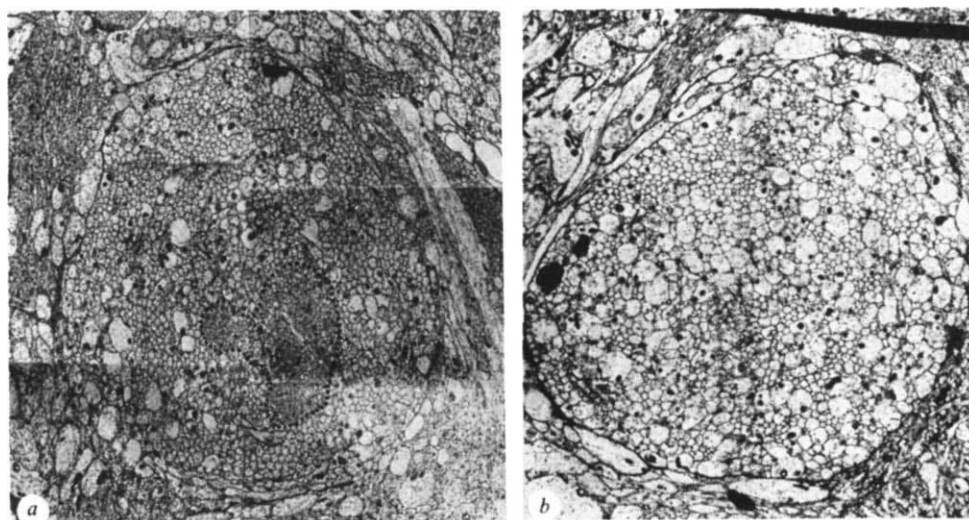


Fig. 3 Cross-section through the caudal peduncle of *a*, late third instar larva of wild type. Note centrally positioned bundle of very thin fibres (encircled by dotted line); *b*, late third instar larva of mutant *mushroom bodies deranged*^{KS65}. During larval life the mushroom bodies have the normal shape. However, in many peduncles, the bundle of thin fibres is missing. $\times 3,245$.

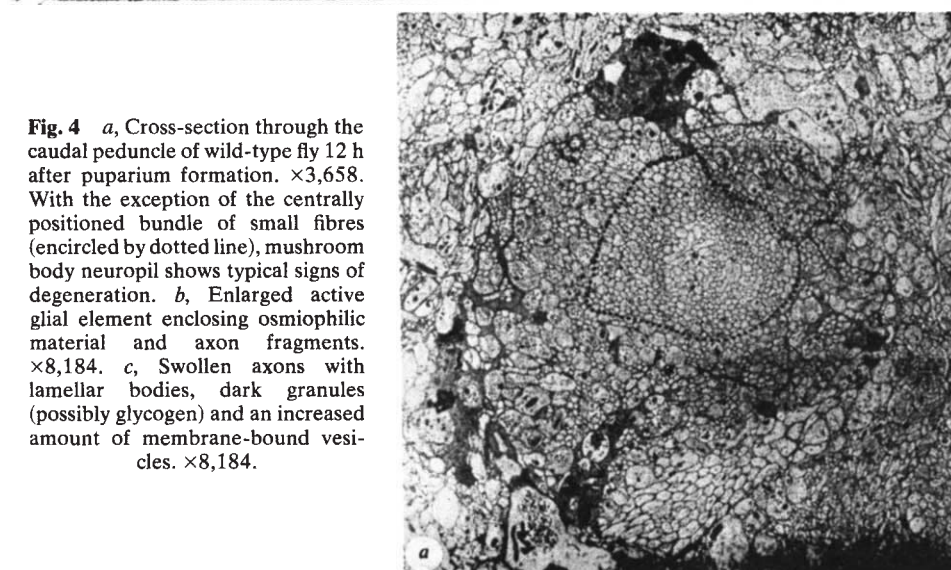


Fig. 4 *a*, Cross-section through the caudal peduncle of wild-type fly 12 h after puparium formation. $\times 3,658$. With the exception of the centrally positioned bundle of small fibres (encircled by dotted line), mushroom body neuropil shows typical signs of degeneration. *b*, Enlarged active glial element enclosing osmiophilic material and axon fragments. $\times 8,184$. *c*, Swollen axons with lamellar bodies, dark granules (possibly glycogen) and an increased amount of membrane-bound vesicles. $\times 8,184$.

functional implications of the reorganization. During pupation, olfactory sense organs are completely demolished and replaced. The AGT fibres must form new connections with the antennal olfactory fibres and this possibly requires new connections between AGT fibres and Kenyon cells as well. Thus, the reorganization of the mushroom bodies may be directly related to the reorganization in the periphery. On the other hand, the presumed different synaptic contacts of the Kenyon cells may also be related to the different 'meaning' some odours have for larva and imago⁹.

The reorganization of the mushroom bodies is the most extensive neuronal rewiring during metamorphosis found to date. We do not yet know whether it is characteristic of mushroom bodies in other insects, or whether other insect brain areas are also reorganized in such a wholesale manner.

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Co-occurrence of substance P-like and Leu-enkephalin-like immunoreactivities in neurones and fibres of avian nervous system

Jonathan T. Erichsen, Anton Reiner & Harvey J. Karten

Department of Neurobiology and Behavior, State University of New York at Stony Brook, Stony Brook, New York 11794, USA

Neuropeptides are frequently found in neurones that also contain another putative transmitter substance such as a biogenic amine or acetylcholine^{1–5}. Such co-existence has led to the suggestion that the roles of neuropeptides in the nervous system may be diverse and may in some systems include a part in conventional neurotransmission as well as an ancillary, or modulatory, role. Recently, several studies have noted the co-occurrence of two peptides within individual neurones of localized regions of the mammalian nervous system^{6–9}. Here, we present the first evidence, based on an indirect immunofluorescence technique allowing the simultaneous visualization of separate tissue antigens, that substance P (SP) and Leu-enkephalin (L-Enk) co-occur within individual neurones of widespread regions of the avian central nervous system.

The most widely used^{3,4,10} immunohistochemical technique for double-label experiments has been the elution technique of Tramu *et al.*¹⁰. However, this technique requires comparisons of the photographic record of individual neurones following staining for each antigen (according to the indirect immunofluorescence method of Coons¹¹) to determine whether any neurone has stained for the presence of both antigens. (An alternative technique involves the comparison of individual neurones in adjacent thin sections (10 μ m) which have been processed separately for the presence of the two given substances^{1,2,6,7}.) In the present approach, immunoreactivity of two separate tissue antigens was visualized simultaneously by using non-cross-reactive primary antisera for SP and L-Enk and two distinct secondary antisera, each of which was specific for only one of the two primary antisera (see Fig. 1 legend). Furthermore, because the terminal fluorescent markers for each tissue antigen were distinct fluorophores, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC), the location of each antigen could be readily determined. The tissue was examined for the two fluorophores using a Leitz epi-illumina-

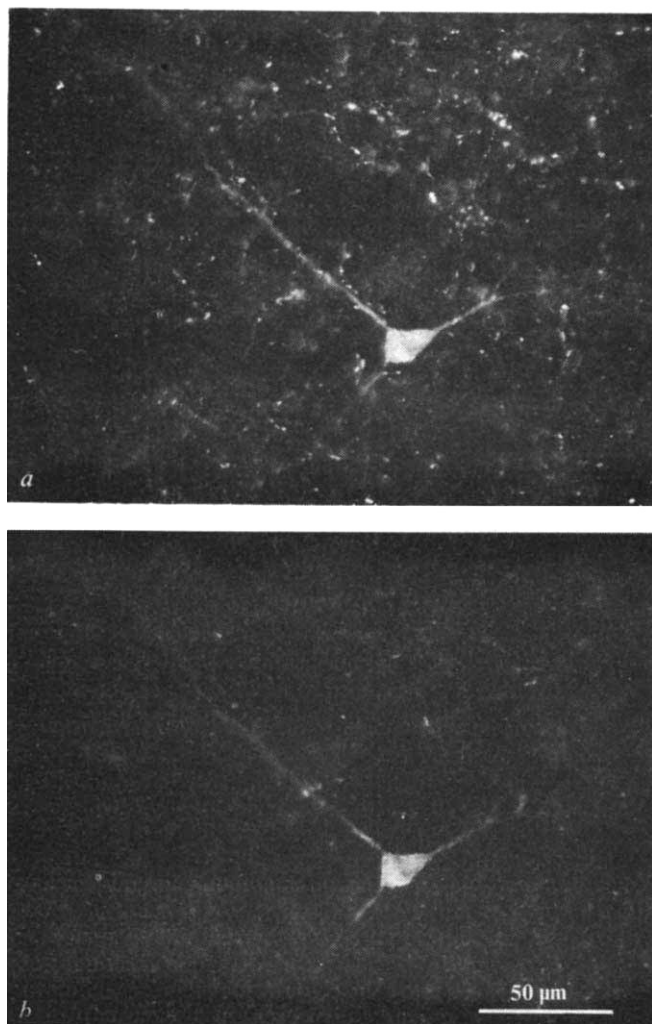


Fig. 1 Photomicrographs of an ICo neurone taken using an N cube (a) and an I cube (b) (note that different labelled fibres are seen through the two different cubes). This neurone showed TRITC (a) and FITC (b) fluorescence, indicating that the neurone stained for the presence of both a L-Enk-like substance (a) and a SP-like substance (b). The L-Enk antiserum (supplied by Dr K.-J. Chang, Wellcome) used was raised in rabbit while the monoclonal SP antiserum (Sera-Lab) used was raised in tissue culture from rat spleen hybridoma. To stain for the presence of the L-Enk and SP antisera, respectively, a goat anti-rabbit IgG conjugated to TRITC (Cappel) and a goat anti-rat IgG conjugated to FITC (Cappel) were used. As neither primary antiserum cross-reacts with either the other primary antiserum or the antigen against which the other primary antiserum is directed and as neither secondary antiserum cross-reacts with either the other secondary antiserum or the primary antigen against which the other secondary antiserum is directed, it proved possible to stain neural tissue simultaneously for the presence of both antigens.

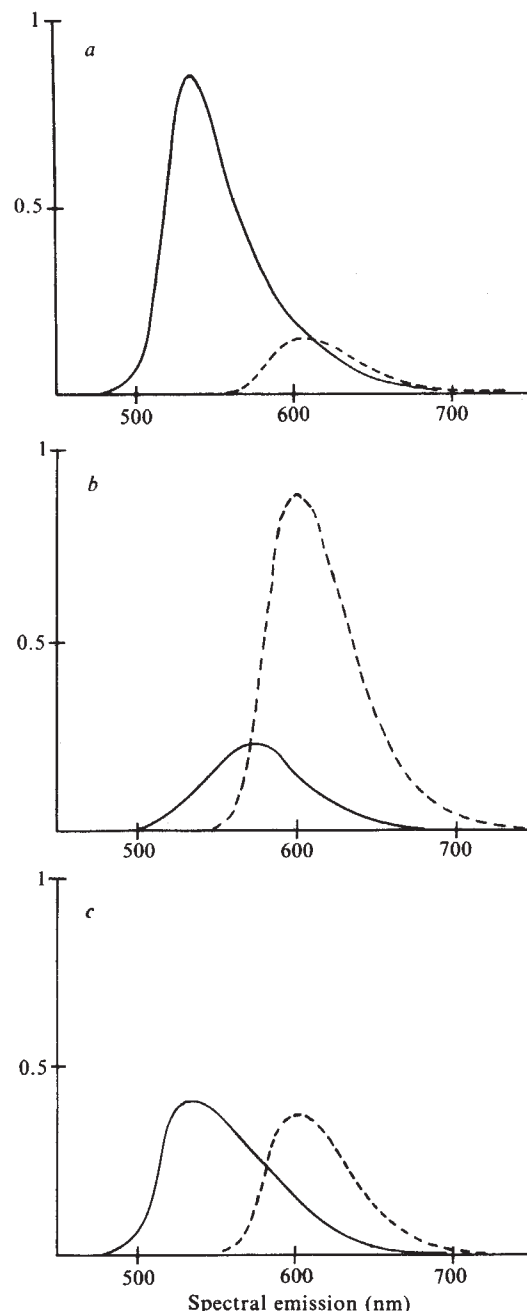


Fig. 2 Emission spectra of neurones stained with FITC- and/or TRITC-conjugated antisera. Although the wavelength bandpass characteristics of the N cube ensured that FITC fluorescence was not detectable while the tissue was being examined using this cube, TRITC fluorescence was observable through the I cube. Nonetheless, because the TRITC fluorescence seen through the I cube is discernibly reddish while FITC fluorescence is greenish-yellow, true FITC fluorescence can be distinguished from TRITC 'break-through'. However, microspectrophotometry (using a Leitz MPV-2 with a B-60 Veril motor-driven filter with output recorded on an HP 2000-A x-y recorder) was used to confirm that individual neurones appearing to show both FITC and TRITC fluorescence did indeed show peak emissions through the N and I cubes at the wavelengths characteristic of TRITC and FITC, respectively. *a* Shows the spectral emission of a neurone from control series 2 (see text) that showed only FITC labelling (solid line represents emission spectrum through I cube; dashed line represents emission spectrum through N cube). The small emission recorded through the N cube represents nonspecific background binding of the anti-rabbit IgG-conjugated TRITC, respectively. *b* Shows the spectral emission of a neurone from control series 3 that showed only TRITC labelling. Note that the peak recorded through the I cube is somewhat displaced to the right of the FITC wavelength peak seen in *a*. This peak represents TRITC breakthrough on the I cube and, as can be seen, has its maximum amplitude in the red range. *c* Shows the emission spectra of the double-labelled ICo neurone shown in Fig. 1. Note that this neurone shows an emission maximum through the N cube at the TRITC peak (see *b*) and an emission maximum through the I cube at the FITC peak (see *a*). The gradations along the y-axis represent an arbitrary scale designed to aid comparisons of the intensity of emission at different wavelengths (x-axis) for different cells.

tion fluorescence microscopy system in conjunction with an 'I' cube for FITC and an 'N' cube for TRITC (Fig. 2). Control tissue incubations were carried out to confirm the specificity of the individual primary and secondary antisera.

Details of the immunohistochemical procedures used have been described elsewhere¹²⁻¹⁴. Tissue incubations were carried out in a primary antisera 'cocktail' consisting of both SP and L-Enk antisera at 1:1,000 dilutions; subsequently, the tissue was incubated in a secondary antisera cocktail consisting of anti-rabbit IgG-conjugated TRITC at a 1:30 dilution and anti-rat IgG-conjugated FITC at a 1:50 dilution. In some cases, three further series of sections from a given animal were run through separate control procedures: (1) incubation in a SP/L-Enk primary antisera cocktail that also contained synthetic SP and L-Enk, (2) incubation only in SP primary antiserum, and (3) incubation only in L-Enk primary antiserum. All three series of sections were subsequently incubated in the above described secondary antisera cocktail. The results of these control procedures were consistent from case to case. In the first (or blocked control) series, no labelled neurones or fibres were observed in the sections, in the second series only FITC-labelled cells and fibres were observed, and in the third control series only TRITC-labelled cells and fibres were observed (Fig. 2). The results of the first control procedure indicated that the individual primary antisera are specific for SP and L-Enk or structurally similar compounds. The specificity of the SP and L-Enk antisera in avian nervous tissue in our experimental conditions has been routinely and consistently observed in many previous single-label studies in our laboratory^{12,13,15-18}. In such studies, as in the present double-label study, no immunoreactivity with either SP antiserum blocked with synthetic SP or L-Enk antiserum blocked with synthetic L-Enk was ever observed in neural regions of interest to the present study. The results of the second and third control procedures show that neither secondary antiserum observably cross-reacts with either opposing primary or secondary antisera. Together, the three control procedures indicate that the immunoreactive substances observed in our double-label experiments are SP and L-Enk or structurally similar compounds. In light of the consistent control results obtained, control procedures were not routinely run with each experimental series.

Separate observations of L-Enk-like and SP-like immunoreactive preganglionic terminal endings in the avian ciliary ganglion¹⁵ suggested that some of the preganglionic terminals within the ciliary ganglion might contain both SP-like and L-Enk-like immunoreactivities, and we have now confirmed this in individual terminal boutons using the double-label techniques described above. Careful examination of double-labelled sections through several levels of the ciliary ganglion indicated that most preganglionic terminal endings in the ciliary ganglion contain both SP-like and L-Enk-like immunoreactivities (Fig. 3), although some terminals contain only a L-Enk-like or SP-like substance. Erichsen *et al.*¹⁶ have shown that lesions of the nucleus of Edinger-Westphal (EW, also known in birds as the accessory oculomotor nucleus) eliminate both the SP-like and L-Enk-like immunoreactivities from the preganglionic terminal endings of the ipsilateral ciliary ganglion, thus indicating that EW contains the cell bodies of origin of the L-Enk-like and SP-like immunoreactive terminal endings in the ciliary ganglion. In birds given a 3 μ l injection of colchicine (6 mg μ l⁻¹) into the lateral ventricle 48 h before death, examination of the fluorescent labelling of EW neurones indicated the presence of numerous cell bodies containing both SP-like and L-Enk-like immunoreactivities, as well as neurones that showed immunoreactivity for only SP or L-Enk.

Extensive searches were performed in several other regions of the avian brain known from preliminary observations and published work^{17,18} to contain large numbers of neurones showing specific SP-like and specific L-Enk-like immunoreactivities. These were: (1) the small-celled zone of the basal ganglia (comprising the lobus parolfactorius and palaeostriatum augmentatum), (2) the mesencephalic subventricular nucleus

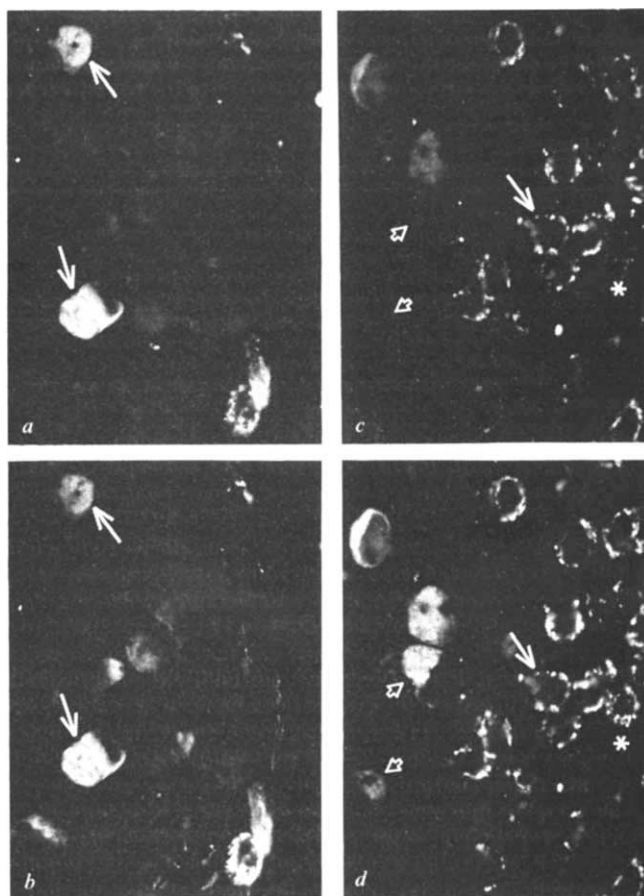


Fig. 3 Photomicrographs of labelled preganglionic terminals in the avian ciliary ganglion. The two types of preganglionic terminals previously described in the avian ciliary ganglion²³ are large cap-like structures that envelop the hilar pole of postganglionic neurones reported to project exclusively to the iris sphincter muscle and the ciliary body and endings characteristically consisting of numerous beaded terminal boutons encircling postganglionic neurones that uniquely project to the smooth musculature of the chorio-capillaris. Both types of terminal endings generally contained both a SP-like substance and a L-Enk-like substance, although the amount of immunofluorescence observed for SP relative to L-Enk varied from terminal to terminal in a given section. *a* and *b* are of the same field of view as seen through an N cube (*a*) and an I cube (*b*). Through the level of the ciliary ganglion shown in *a* and *b*, mostly cap-like endings are present and two of these (indicated by large solid arrows) show both strong TRITC immunofluorescence (indicating the presence of a L-Enk-like substance) and strong FITC immunofluorescence (indicating the presence of a SP-like substance). Several other cap-like endings showing varying amounts of both SP-like and L-Enk-like immunofluorescence are also present in *a* and *b*. One cap-like ending to the lower left in *b* and some fibres to the lower right apparently show only SP-like immunofluorescence. *c* and *d* show the same field of view through the ciliary ganglion as seen through an N cube (*c*) and an I cube (*d*). The level of the ciliary ganglion shown in *c* and *d* contains both cap-like and bouton-like terminal endings. Nearly all bouton-like endings show both TRITC and FITC immunofluorescence (one such double-labelled terminal ending is indicated by the large solid arrows in *c* and *d*), although variations occur in the relative amounts of TRITC and FITC immunofluorescence (for example, bouton-like endings immediately above the asterisk in *c* and *d* show large amounts of SP immunofluorescence but little L-Enk immunofluorescence). The open arrows in *c* and *d* indicate a pair of cap-like endings that both show a large amount of SP-like immunoreactivity (*c*) but little to no observable L-Enk-like immunoreactivity (*d*).

intercollicularis (ICo), (3) the hypothalamus and (4) the periaqueductal grey. Immediately rostral to the confluence of the tectal ventricle with the cerebral aqueduct, we observed many ICo neurones that contained both SP-like and L-Enk-like immunoreactivities (Fig. 1). However, no neurones showing the co-existence of a SP-like and L-Enk-like substance were seen at any other level of ICo, although all levels of ICo contained neurones showing only SP-like or only L-Enk-like immunoreactivity. A population comprising neurones whose cell bodies contained both SP-like and L-Enk-like immunoreactivities and also many neurones whose cell bodies showed only SP-like or L-Enk-like immunoreactivity was found

in a distinct, round cell group situated dorsally in a portion of the avian hypothalamus (or ventral thalamus) termed the stratum cellulare internum (SCI)¹⁹. A few neurones in the periaqueductal grey also contained both a SP-like and a L-Enk-like substance, but no such cells were found on examination of ~1,000 FITC-labelled and ~1,000 TRITC-labelled neurones in the basal ganglia.

The present demonstration of the co-existence of a SP-like substance and a L-Enk-like substance in individual neurones and/or fibre terminals in at least five separate regions of the avian nervous system raises the possibility that the co-existence of substance P and an opioid peptide in individual neurones and fibres may be a widespread phenomenon in the nervous system of a variety of vertebrates, including mammals. Consistent with this, a subpopulation of glomus cells of the carotid body of the cat has also recently been found to contain both SP-like and L-Enk-like immunoreactive material (L. Stensaas, personal communication). The functions of SP and L-Enk in neurones and fibres where they co-exist are unclear. In systems studied where they occur singly, SP and L-Enk have opposite actions, the former having excitatory postsynaptic effects and the latter inhibitory postsynaptic effects²⁰. Clearly, however, the two need not have the same roles in systems where they co-occur as in those where they occur singly.

As the neurones of EW that project to the ciliary ganglion (a parasympathetic ganglion) are reported to be cholinergic^{21,22}, the present data suggest the co-existence of SP and L-Enk in a population of cholinergic neurones. Further, our results provide evidence that numerous individual preganglionic terminal boutons of the ciliary ganglion also contain both SP and L-Enk, and presumably acetylcholine. Erichsen *et al.*¹⁵, using electron microscopic immunohistochemical techniques to study separately the ultrastructural localization of SP-like and L-Enk-like immunoreactivities in the preganglionic terminals of the ciliary ganglion, found that both SP-like and L-Enk-like immunoreactivities occurred in the same size class of large dense-core vesicles (85 nm, mean diameter). However, the resolution of the present light microscopic double-label technique was insufficient to determine whether SP-like and L-Enk-like immunoreactivities occurred in the same individual vesicles. Nonetheless, their presence in presumptively cholinergic preganglionic terminal boutons in the ciliary ganglion suggests that all three may have a role in neurotransmission in the ciliary ganglion.

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Rapid kinetics of single glutamate-receptor channels

S. G. Cull-Candy & I. Parker

Department of Biophysics, University College London, Gower Street, London WC1E 6BT, UK

Drug-induced ionic channels are known to operate by opening briefly when agonist molecules bind to receptor sites^{1–7} to produce rectangular current pulses, the amplitude and lifetime of which can be derived from noise analysis^{1–3}, or directly recorded using the patch-clamp technique^{4–7}. If desensitization is neglected, the distributions of channel open and closed times can generally be fitted by single exponential functions^{5–7}, suggesting that a simple two-stage reaction scheme is sufficient to account for the observed kinetics¹. Using patch-clamp recording with an improved time resolution, we have now found that glutamate-activated channels in locust muscle membrane show a large excess of both brief openings and closings over those expected from single exponential distributions. Many events which, with poorer frequency resolution, would have appeared as single openings were found to consist of two or more openings, interspersed with brief (<300 μ s) closings. These observations have interesting implications for receptor/channel functioning.

Patch-clamp recordings of single glutamate-activated channels were obtained from the extensor tibiae muscle of the locust (*Schistocerca gregaria*), using techniques similar to those described previously^{4,6}. Patch-clamp pipettes contained 200 μ M glutamate, which gave a high frequency of channel openings, and muscle fibres were usually voltage-clamped at a potential of -110 mV to increase the size of the single channel currents. Muscles were pretreated with 1 μ M concanavalin A to reduce receptor desensitization⁵, and recording patches were

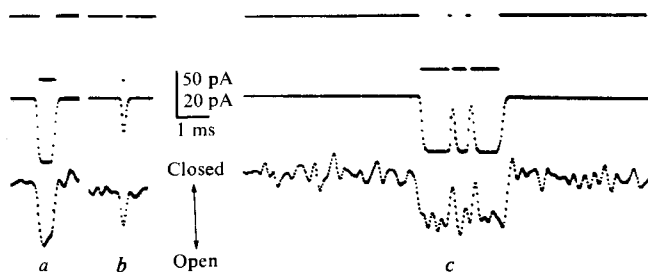


Fig. 1 Patch-clamp recordings of test pulses (*a, b*) and single channel currents (*c*) illustrating the time resolution of the recording system and the method of measurement. In each record the lower trace is a computer-digitized signal from the patch-clamp amplifier, sampled at 25- μ s intervals after sharp cut filtering ($1/f^4$) at 3.2 kHz. The upper traces show computer-generated traces used to help measure the data. A rectangular pulse (upper trace) with a height equivalent to the single channel current was generated and the position and width of this could be altered manually. A smoothing function applied to this pulse approximated the step response function of the recording system and the resultant smoothed pulse (middle trace) was displayed on a screen superimposed on the data. The position and width of the generated rectangular pulse was adjusted so that the smoothed pulse gave the best match to a channel opening and these parameters were then stored. Channel openings in each 512-point data block were fitted sequentially and a display of previously fitted openings was maintained to assist in matching brief channel closings (*c*). After analysis of several hundred data blocks, histograms of open and closed times of the generated rectangular pulses were automatically constructed. *a, b*, Lower traces show recordings of rectangular test pulses with durations of 500 and 50 μ s respectively. Upper traces are computer-generated pulses fitted to the recordings. Calibrations, 50 pA and 1 ms. *c*, Lower trace shows single channel currents recorded from a membrane patch. Channel openings are shown as downward deflections. Upper trace shows the rectangular current pulses considered to underlie the records, whilst the middle trace shows the expected distortion of these pulses due to the frequency response characteristics of the recording system. Calibrations, 20 pA and 1 ms.

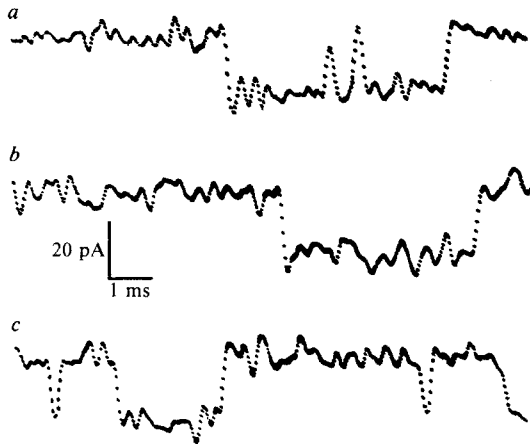


Fig. 2 Examples of single channel currents recorded with high time resolution (d.c. to 3.2 kHz). Channel openings correspond to downward deflections. Same membrane patch and recording parameters as in Fig. 1c. Temperature, 23 °C. Calibrations, 20 pA and 1 ms.

selected where only one active channel was present (that is, where no simultaneous openings were observed⁶). The time resolution of the recordings was improved significantly by using a circuit, after the headstage amplifier, which partially compensated for high-frequency roll-off caused by capacitance across the feedback resistor. This gave an overall frequency response of d.c. to ~5 kHz, but records were usually filtered at 3 kHz to reduce noise, before being digitized for computer analysis. Data were sampled in blocks of 512 points, at a digitization rate of 40 or 20 kHz.

The frequency response of the patch clamp was such that test pulses lasting 500 μ s could be recorded with little distortion (Fig. 1a), whilst 50 μ s pulses were detectable but became broadened and attenuated (Fig. 1b). The rise and fall times of single channel currents were similar to those of the test pulses (Figs 1c, 2), suggesting that the true transition times of the channel were faster than the resolution of the recording system. To permit more accurate measurements of brief channel openings and closings, a computer program was developed which partially compensated for the restricted frequency response of the recordings (see Fig. 1 legend). The ultimate limiting factor in time resolution was then set by the signal-to-noise ratio. We were unable to obtain seal resistances against the muscle membrane of better than 20 M Ω , so noise from this source was high⁴, but as large single channel currents (20 pA) can be recorded from locust muscle the problem was not too serious.

A striking feature in our records was that many channel openings were interrupted by one or more brief closings (Figs 1c, 2a), although some long openings showed no apparent intervening closings (Fig. 2c). Also, there seemed to be more brief openings than expected from an exponential distribution (Fig. 2c). A quantitative examination was made by forming histograms of open and closed time distributions; Fig. 3 illustrates data from 832 channel openings recorded from one membrane patch. The present results are consistent with previous findings that both the open and closed time distributions of single glutamate channels can be fitted reasonably by single exponentials^{5,6} and that open times are exponentially distributed³, except that at short intervals (<200 μ s) there is a considerable excess of both channel openings and closings (Fig. 3b, d), which are probably too brief to have been detected in previous patch-clamp studies having a narrower frequency range.

Figure 3a, c shows the distributions of brief openings and closings on an expanded time scale. In both cases an excess of events over a single exponential distribution becomes apparent at intervals of <~300 μ s, and increases steeply with shorter intervals, except for a falloff in the shortest histogram bin (0–50 μ s). A duration of 50 μ s is about the limit of resolution of

the recording system, so the falloff in this bin is probably due to very brief events remaining undetected.

We attempted to estimate roughly the total numbers of brief openings and closings by assuming they are distributed exponentially, and fitting the observed distributions by the sum of two exponentials corresponding to the 'brief' and 'normal' events. In the case of channel closings, the data (Fig. 3c) can be fitted by exponentials with time constants $\tau_{\text{fast}} = 100 \mu\text{s}$, $\tau_{\text{slow}} = 2 \text{ ms}$. The predicted total excess of brief closings (=area under fast exponential) is 383, compared with 616 closings fitted by the slow exponential. It is clear therefore that channel openings generally occurred in bursts, with brief intervening closings within bursts and, on average, longer closings between bursts. For the events in Fig. 3 the mean number of closings per burst is

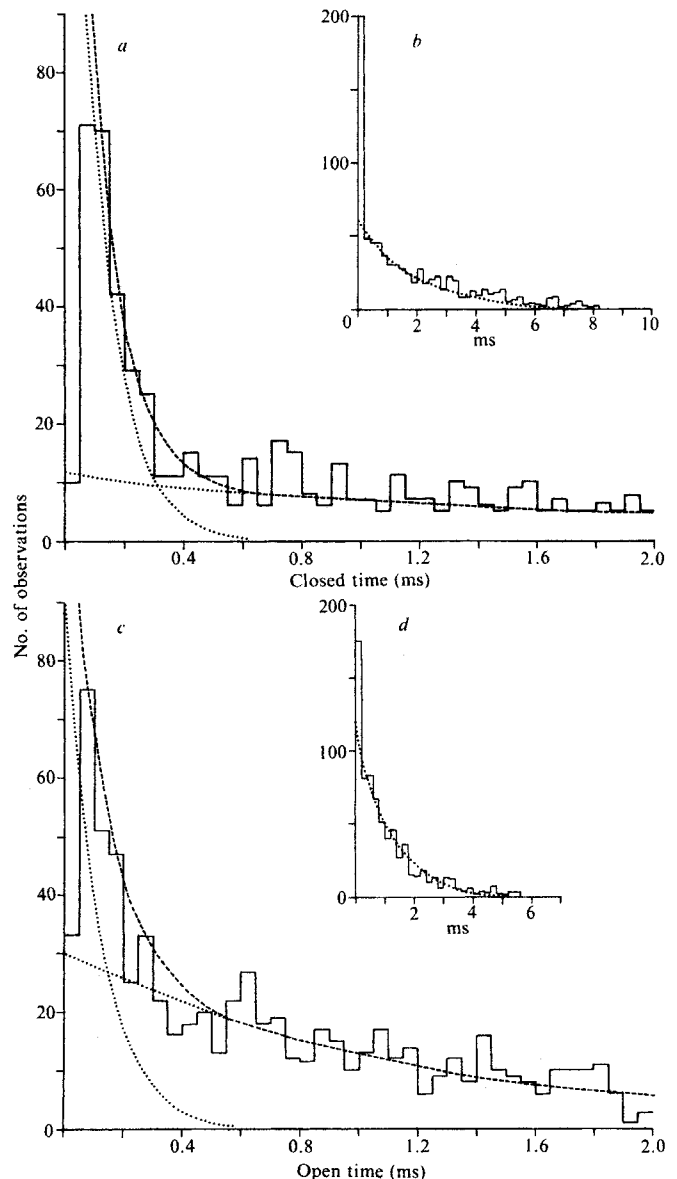


Fig. 3 Histograms of distributions of single channel closed (a, b) and open (c, d) times illustrating the excess of brief events. Data are measurements from 832 channel openings recorded from one membrane patch where only one channel appeared to be present. During the entire recording period the channel was in the 'normal' kinetic state⁶. Insets (b, d) show the same data as the main histograms, but on a different time scale. The distributions in a and c have been manually fitted by the sum of two exponentials; the component exponentials are indicated by dotted curves and the sum by dashed curves. Only the slow exponentials are shown in the insets. Time constants of the exponentials are: open times, $\tau_{\text{fast}} = 120 \mu\text{s}$, $\tau_{\text{slow}} = 1.5 \text{ ms}$; closed times, $\tau_{\text{fast}} = 100 \mu\text{s}$, $\tau_{\text{slow}} = 2 \text{ ms}$. Area under the exponentials are: open time, fast = 220, slow = 717; closed time, fast = 383, slow = 616. In preliminary experiments with different glutamate concentrations, estimated time constants for the closed time were: 100 μM , $\tau_{\text{fast}} = 110 \mu\text{s}$, $\tau_{\text{slow}} = 6.8 \text{ ms}$; 600 μM , $\tau_{\text{fast}} = 117 \mu\text{s}$, $\tau_{\text{slow}} = 0.86 \text{ ms}$ (mean values from 5–7 patches).

~0.6 and hence the mean number of openings per burst is 1.6. The mean open time per burst can be estimated from the total area under the open time distribution (dashed line in Fig. 3a) divided by the number of inter-burst intervals and is 1.64 ms. Hence, the total burst length is ~1.7 ms and the mean duration of the openings within a burst ~1.0 ms. This estimate includes both the fast and slow components of the open time distribution. If the brief intra-burst closings were not detected, the apparent 'channel lifetime' would correspond to the mean burst length, and previous estimates of channel lifetime from noise analysis³ and patch-clamp recording^{5,6} are in reasonable agreement with our estimate of the mean burst length.

Considering next the excess of brief openings, the mean lifetime of this component estimated from the fitted fast exponential is 120 μ s, and the predicted number in the recording period is 220, compared with 717 'normal' openings. Thus, about one-quarter of all channel openings were due to the brief component, but because of their short duration they would have carried only ~3% of the total current.

Records from seven other membrane patches (four muscles) using 200 μ M glutamate were similar: a mean value of 1.6 ± 0.03 (s.e.) openings per burst, with a mean channel open lifetime of 0.97 ± 0.12 ms. However, in view of possible errors and assumptions in fitting the fast exponential functions to the tails of poorly resolved distributions, these values should be regarded as provisional.

Brief intervening closings during channel openings and an excess of brief openings have been observed from acetylcholine-receptor channels in frog twitch muscle⁸ and the existence of intra-burst closings has been inferred from noise analysis in frog slow muscle fibres⁹. It seems likely therefore that these features may be general to most drug-activated channels. One possible mechanism for the intra-burst closings is that the channel is transiently blocked in a manner analogous to the action of local anaesthetics^{10,11}. If this blocking resulted from glutamate molecules plugging the channel, then the number of brief closings would be expected to increase directly with glutamate concentration. In preliminary experiments we found that the number of brief closings per millisecond of open time showed little change over a range of glutamate concentrations (100, 200, 400, 600 μ M). A more attractive possibility, which has been predicted from model reaction schemes^{12,13}, is that the intra-burst closings result from re-isomerization of the channel to the open configuration after closing, without dissociation of the bound agonist molecules. A simple two-step model for channel opening is:



where A = agonist molecule, T = closed channel and R = open channel. In this scheme, intra-burst closings result from the transitions $AR \rightarrow AT \rightarrow AR$, and their mean duration is $1/(\beta + k_{-1})$. For our data $\beta \approx 4 \times 10^3 \text{ s}^{-1}$. Similarly, $\alpha \approx 1/\text{channel lifetime} \approx 10^3 \text{ s}^{-1}$. The mean number of openings per observed burst $= (1 + \beta/k_{-1}) = 1.6$; hence $k_{-1} \approx 6 \times 10^3 \text{ s}^{-1}$. In fact, this two-step model is probably an over-simplification; for example, opening of the glutamate channel seems to involve binding of two or more agonist molecules^{6,14}. Such a cooperative model is expected to give rise to a three-component distribution of closed times¹³. The intermediate component (due to brief closings via the singly bound state) may be partially included in our estimated fast component, but calculations based on a cooperative model indicate that the maximum contribution would be smaller than our experimental error. The rough estimates of α , β and k_{-1} are therefore applicable to both cooperative and non-cooperative models.

The origin of the excess brief openings is more mysterious. One possibility, which may be readily discarded, is that two separate channels of different mean lifetimes, were present under the patch pipette. If this were the case, 47 simultaneous openings would have been expected in the record of Fig. 3, and the probability of observing zero simultaneous events is $\approx 10^{-20}$;

none was observed. Another possibility, which has been suggested to explain similar brief openings of acetylcholine-receptor channels⁸, is that they arise from channel openings when only one agonist molecule is bound. Important clues as to the origins of both the brief openings and closings should be given by their dependence on agonist concentration and species of agonist. It is hoped to explore these possibilities further using internally perfused patch-clamp pipettes^{5,14}.

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T-cell colonies recognize antigen in association with specific epitopes on Ia molecules

Robert B. Clark, Joe Chiba, Stephen E. Zweig & Ethan M. Shevach

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205, USA

The products of the *I* region of the major histocompatibility complex have a role in many immunological processes and are intimately involved with, if not identical to, the genes controlling specific immune responses (*Ir*). Alloantibodies to *I* region-associated (Ia) antigens have proved extremely useful in defining the relationship between Ia antigens and *Ir* gene product function¹. We have recently reported data which suggest that monoclonal anti-Ia antibodies interact with different epitopes (antigenic determinants) on macrophage Ia molecules and that distinct epitopes, though on the same Ia molecule, are recognized by T cells in association with different antigens or antigen fragments². We have now used these monoclonal xenogeneic antibodies to guinea pig Ia antigens together with antigen-specific T-cell colonies to analyse further the role of macrophage Ia antigens in T-cell activation. We demonstrate here that the proliferative responses of individual antigen-specific T-cell colonies were often completely inhibited by only one of several anti-Ia monoclonal antibodies. These results strongly support the notion that monoclonal anti-Ia antibodies can be used to define epitopes on Ia molecules that have unique functions in the activation of antigen-specific T cells, and that individual T-cell colonies recognize antigen in association with functionally distinct epitopes on Ia molecules.

Antigen-specific T-cell colonies were prepared from lymph node cells of guinea pigs primed *in vivo*, using a modification of the soft agar cloning technique described elsewhere³. Over 80% of the colonies that were picked from the agar could subsequently be expanded to testable numbers of cells, and ~95% of these colonies proliferated specifically when challenged with the soluble protein antigens used to generate the colonies, but not in response to other antigens to which the T-cell donor had been primed (data not shown). Furthermore, it is likely that the initial colonies picked from soft agar represent

Table 1 Antigen-specific MHC-restricted guinea pig T-cell colonies

Colony	T cell	Macrophage used to generate colony	Antigen	Δ c.p.m. ^3H -TdR incorporation into test culture Macrophages		
				Strain 2	Strain 13	(2 × 13)
10	(2 × 13)F ₁	(2 × 13)F ₁	PPD	61	5,705	2,216
31	(2 × 13)F ₁	(2 × 13)F ₁	PPD	319	16,333	13,450
124	(2 × 13)F ₁	(2 × 13)F ₁	OVA	<10	7,301	12,896
126	(2 × 13)F ₁	(2 × 13)F ₁	OVA	3,170	958	4,998
62	(2 × 13)F ₁	Strain 2	PPD	9,126	435	8,582
63	(2 × 13)F ₁	Strain 2	PPD	18,519	<10	13,613
131	(2 × 13)F ₁	Strain 2	PPD	21,070	907	ND
500	Strain 13	Strain 13	PPD	148	9,027	12,537
501	Strain 13	Strain 13	PPD	<10	13,391	13,503

Antigen-specific T-cell colonies were generated from inbred strain 2, strain 13 or (2 × 13)F₁ regional lymph node nylon wool-purified T cells obtained from animals which had been primed *in vivo* with ovalbumin (OVA) (Miles) in complete Freund's adjuvant (CFA) (Difco). T cells (4×10^6) were cultured with X-ray irradiated (2,500 R) non-immune, unfractionated mineral oil-induced PECs (1×10^6) and 100 $\mu\text{g ml}^{-1}$ PPD (Connaught) or OVA for 4–7 days in 24-well plates (Costar) in RPMI-1640 (Gibco) containing 300 $\mu\text{g ml}^{-1}$ L-glutamine, 100 U ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin, 1 $\mu\text{g ml}^{-1}$ 5-fluorocytosine, 5×10^{-5} M 2-mercaptoethanol (complete medium), and 5% heat-inactivated normal guinea pig serum. After this period of liquid culture, the antigen-stimulated T cells were plated onto a double layer of soft agar. The lower layer of agar, prepared 24 h before the upper layer, consisted of 0.5% Noble agar (Difco), 100–300 $\mu\text{g ml}^{-1}$ PPD or OVA, with or without 25% T-cell growth factor (TCGF) and 10% heat-inactivated fetal calf serum (FCS). The TCGF used consisted of crude supernatants of concanavalin A-stimulated guinea pig lymph node cells and always contained 20 mg ml^{-1} α -methylmannoside (Calbiochem-Behring). The upper layer of agar consisted of 0.32% Noble agar, 10% heat-inactivated FCS, fresh PECs (2×10^5) and antigen-stimulated T cells ($4\text{--}8 \times 10^5$). After 3–5 days on agar, colonies consisting of ~50 cells were picked off with a Pasteur pipette and then liquid-cultured in complete medium with 5% heat-inactivated FCS, 100 $\mu\text{g ml}^{-1}$ PPD or OVA, 10^4 PECs and 25% TCGF in 96-well flat-bottomed microtitre plates (Costar). Expanding colonies were transferred to 24-well plates and fed twice weekly with 5×10^5 PECs, 200 $\mu\text{g ml}^{-1}$ PPD or OVA and 25% TCGF. Antigen-induced proliferation of T-cell colonies (10^4) was measured by incubation with 10^5 PECs and 100 $\mu\text{g ml}^{-1}$ PPD or OVA in 0.2 ml of complete medium with 5% heat-inactivated FCS and 10% TCGF in flat-bottomed microtitre wells. After 3 days, the contents of each well were divided into three new flat-bottomed, microtitre plates, each containing 0.2 ml of complete medium with 5% heat-inactivated FCS, 10% TCGF, 100 $\mu\text{g ml}^{-1}$ PPD or OVA and fresh PECs (10^5). After an additional 48 h of culture, 1.0 μCi of tritiated thymidine (^3H -TDR: specific activity 6.7 Ci mmol^{-1} ; NEN) was added to each well and after 18 h the amount of radioactivity incorporated into the cellular DNA was determined using a semi-automated microharvesting device (Mash II; Microbiological Associates). The results of triplicate cultures are expressed as total c.p.m. with antigen – c.p.m. without antigen (Δ c.p.m.). The s.e.m. of triplicate samples was rarely >10% of the mean, and for simplicity, only the mean of triplicate samples is given. ND, not determined.

ted true T-cell clones and not mixed cell populations because almost all T-cell colonies derived from (2 × 13)F₁ donors that had been selected and expanded with F₁ peritoneal exudate cells (PECs) demonstrated an antigen-specific proliferative response in the presence of one but not the other parental strain of PEC (Table 1, colonies 10, 31, 124 and 126). (2 × 13)F₁ T-cell colonies that had been generated and expanded with strain 2 PECs, and T-cell colonies derived from inbred strain 13 T cells that had been expanded with syngeneic PECs, also demonstrated a strict genetic restriction in their proliferative response to the appropriate strain of PEC (Table 1, colonies 62, 63, 131, 500 and 501).

The production and characterization of the murine monoclonal antibodies to guinea pig Ia antigens have been described in detail elsewhere⁴. Both monoclonals 22C4 and 27E7 reacted with all Ia antigens identified by a strain 2 anti-strain 13 serum (anti-Ia 1, 3, 7). However, in sequential immunoprecipitation studies with a strain 13 anti-strain 2 serum (anti-Ia 2, 4), monoclonal 27E7 reacted only with the Ia 2-bearing molecule while monoclonal 22C4 reacted with all the Ia molecules identified by the anti-Ia 2, 4 serum (ref. 4 and S.E.Z. and E.M.S., in preparation). Monoclonal antibodies 25E11 and 13S1 reacted with alloantigenic determinants of strain 2 and strain 13 Ia molecules, respectively.

These monoclonal antibodies were studied for their effects on the proliferative responses of the antigen-specific T-cell colonies; as a control population we used short-term lines of antigen-specific T cells which had been prepared by stimulation with antigen in liquid culture but which had not been separated into distinct colonies on agar. Colonies 62, 63 and 131 are strain 2-restricted, purified protein derivative of tuberculin (PPD)-specific, (2 × 13)F₁ T-cell colonies. As seen in Table 2, colony 62 was inhibited only by monoclonal antibody 22C4, which is directed against an Ia determinant present on all strain 2 and strain 13 Ia molecules. Colony 63 was inhibited only by monoclonal antibody 25E11, which is directed against a strain 2 alloantigenic determinant. Colony 131 was inhibited by monoclonal antibodies 25E11 and 27E7, the latter being directed against a determinant present on all strain 13 and on some, but not all, strain 2 Ia molecules. Thus, while monoclonal 22C4 reacts with all strain 2 and strain 13 Ia molecules, it inhibits the response of colony 62 but not that of colonies 63 and 131. This indicates that colony 62 recognizes PPD in association with a

region or epitope on the restricting Ia element different from that recognized by colonies 63 and 131. It is also likely that the latter colonies recognize PPD in association with distinct epitopes, as colony 63 was blocked only by 25E11 whereas colony 131 was blocked by both 25E11 and 27E7. As competitive binding studies have demonstrated partial inhibition of the binding of 25E11 by 27E7, the determinants identified by these monoclonal antibodies are probably adjacent to each other on the Ia molecule (S.E.Z. and E.M.S., unpublished observations). Thus, colony 131 may react with PPD in association with an epitope of strain 2 Ia located between the sites defined by 25E11

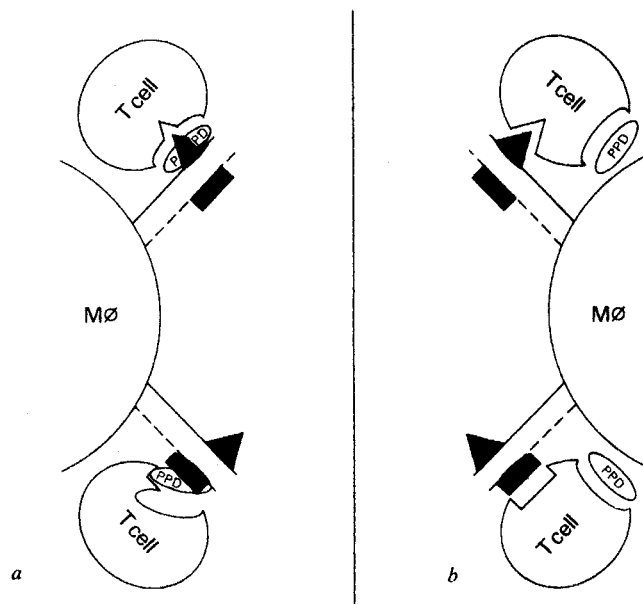


Fig. 1 a, Model of antigen presentation in which distinct epitopes on macrophage Ia molecules interact with conventional antigens and determine the orientation of antigenic determinants presented to the responding T cells. b, Model of antigen presentation in which distinct epitopes on Ia molecules and conventional antigens are recognized separately by two distinct receptors on responding T cells.

Table 2 Effect of monoclonal anti-Ia antibodies on the antigen-specific proliferative responses of T-cell colonies and T-cell lines

Responder	PPD-pulsed macrophage (MØ)	Δ c.p.m. ^3H -TdR incorporation in the presence of monoclonal anti-Ia antibody				
		Control	25E11	27E7	22C4	13S1
Colony 62	Strain 2	2,178	3,906	2,669	643	ND
Colony 63	Strain 2	13,055	863	15,022	12,956	ND
Colony 131	Strain 2	5,785	803	1,089	5,473	ND
T-cell line (2 × 13)F ₁	Strain 2	9,353	4,605	6,184	6,184	6,946
Colony 500	Strain 13	3,190	4,038	182	2,533	2,587
Colony 501	Strain 13	1,361	1,153	795	1,540	19
T-cell line (strain 13)	Strain 13	13,031	13,465	3,105	11,490	1,832

Antigen-specific T-cell colonies were generated as described in Table 1 legend. Short-term T-cell lines were generated as follows: nylon wool column-purified T cells were obtained from regional lymph nodes of inbred strain 13 or (2 × 13)F₁ guinea pigs which had been primed *in vivo* with OVA in CFA. T cells (4×10^6) and irradiated, non-immune mineral oil-induced PECs (1×10^6) were cultured in complete medium containing 5% heat-inactivated normal guinea pig serum and $100 \mu\text{g ml}^{-1}$ PPD for 1 week before testing. (2 × 13)F₁ T cells were cultured with parental strain 2 PECs. Inbred strain 13 T cells were cultured with strain 13 PECs. Antigen-induced proliferation of short-term T-cell lines or antigen-specific T-cell colonies was measured by incubation with antigen-pulsed PECs. For antigen pulsing, unfractionated, mineral oil-induced, non-immune, irradiated PECs ($10 \times 10^6 \text{ ml}^{-1}$) were incubated in complete medium at 37 °C for 1 h in the presence of $100 \mu\text{g ml}^{-1}$ PPD. The cells were then washed three times to remove unbound antigen. These antigen-pulsed PECs (1×10^5) were incubated in 0.2 ml of complete medium with 5% heat-inactivated FCS and 5% TCGF, together with cells from T-cell lines or T-cell colonies (2×10^3) for 3 days in a round-bottomed microtitre plate (Linbro Chemical). To measure inhibition of antigen-specific proliferation by the monoclonal anti-Ia antibodies, ascitic fluids produced from the individual hybrid cell lines were added to the above microtitre wells at a final concentration of 1%. As all the monoclonal antibodies used were either of the IgM or IgG1 isotype⁴, we used as control ascitic fluid, a hybrid myeloma line (provided by Ms R. Lieberman) which secreted an IgM antibody directed against inulin in addition to the parental IgG1 antibody of the P3 × 63Ag8 myeloma line. All the ascites containing monoclonal anti-Ia antibody had previously been found to have peak binding or cytotoxicity values when used at 0.1% or 0.01%. ^3H -TdR (1.0 μCi) was added to each well after 48 h of culture and 18 h later the amount of radioactivity incorporated into cellular DNA was determined using a semi-automated microharvesting device, Mash II. The results of triplicate cultures are expressed as total c.p.m. with antigen-pulsed PECs plus ascites – total c.p.m. with unpulsed PECs plus ascites (Δ c.p.m.). The s.e.m. of triplicate samples was rarely >10% of the mean, thus for simplicity only the mean of triplicate samples is given. ND, not determined.

and 27E7, while colony 63 reacts with PPD in association with an epitope close or identical to that defined by 25E11. Colonies 500 and 501, both derived from PPD-immune strain 13 T cells, were also inhibited by different monoclonal anti-Ia antibodies (Table 2). Despite the fact that monoclonal 27E7 reacts with a determinant present on all strain 13 Ia molecules, colony 500 was significantly inhibited by 27E7 whereas colony 501 was inhibited significantly only by monoclonal 13S1, which is directed against an alloantigenic determinant on strain 13 Ia molecules.

In contrast to the inhibition profiles of the colonies, the PPD-specific response of the short-term line of (2 × 13)F₁ T cells was inhibited only moderately by 25E11, 27E7 and 22C4 (Table 2). The short-term line of inbred strain 13 PPD-specific T cells was significantly inhibited by 27E7 and 13S1 (Table 2). Thus, both short-term T-cell lines demonstrated some inhibition by more than one monoclonal antibody. These results are identical to those observed for T cells stimulated directly after being taken from the animal, without previous short-term culture².

Our results are consistent with the hypothesis that the monoclonal anti-Ia antibodies inhibit T-cell proliferation by masking individual Ia epitopes and that individual antigen-specific T-cell colonies recognize antigen in association with such distinct epitopes on Ia molecules. In agreement with this hypothesis, the unseparated T-cell lines, containing multiple antigen-specific clones, were inhibited by more than one monoclonal antibody, and this inhibition was often only moderate. We have recently demonstrated similar differential inhibition by these monoclonal antibodies of alloreactive T-cell colonies, thus these results are not unique to antigen-specific T-cell colonies⁵. It is very difficult to exclude the possibility that our results actually represent inhibition of T-cell recognition by masking of the entire Ia molecule rather than by masking of distinct epitopes on the same Ia molecule. However, the former explanation seems unlikely because several of the monoclonal antibodies which recognize determinants present on all guinea pig Ia molecules inhibit the response of only some of the colonies. Similarly, the differential inhibition by monoclonal antibodies that recognize monomorphic Ia determinants makes it unlikely that monoclonal anti-Ia antibodies inhibit T-cell proliferation by a generalized capping and shedding of Ia antigens. We have so far been unable to reclone any of our antigen-specific T-cell colonies, but the significance of our demonstration that individual antigen-specific T-cell colonies show fine differences in genetic restriction at the level of Ia epitope recognition, is not

dependent on rigorous proof of the monoclonality of these putative clones.

The results presented here are most consistent with the theories of *Ir* gene function proposed by Rosenthal *et al.*⁶ and Benacerraf⁷, in which Ia molecules on macrophages interact with antigens and determine the orientation of antigenic determinants presented to the responding T cells. In this case, monoclonal anti-Ia antibodies would selectively inhibit T-cell activation by masking one of the sites of interaction of the Ia molecules with conventional antigen (Fig. 1a). However, the results are also compatible with the theories⁸ proposing that the T cell has two receptors, one for self-Ia and the other for conventional antigen. In the latter case (Fig. 1b), monoclonal anti-Ia antibodies would inhibit T-cell proliferation by masking the recognition of individual Ia epitopes seen by the anti-self-Ia receptor on the T cell.

These results have important implications for the relationship between Ia antigens, *Ir* genes and disease susceptibility. As suggested by Schwartz *et al.*⁹, the frequently noted associations between disease susceptibility and HLA antigens in man may, in fact, be associations between disease susceptibility and distinct epitopes on those HLA antigens. Determination of such disease-MHC epitope associations could have great practical and theoretical value by allowing more accurate detection of those individuals at highest risk for developing certain diseases and by furthering our understanding of the relationship between the immune system and disease susceptibility.

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Progesterone monoclonal antibody blocks pregnancy in mice

L. J. Wright, A. Feinstein, R. B. Heap, J. C. Saunders, R. C. Bennett & M.-Y. Wang

ARC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, UK

Production of monoclonal antibodies by hybrid cell lines¹ provides a method potentially superior to conventional techniques for raising antisera² in respect of titre and specificity. Many lines have been prepared³ that secrete antibody to cell-surface and soluble antigens and to viruses, but the products of hybridomas that recognize steroid haptens have not yet been exploited. Progesterone, the hormone of pregnancy, is essential for the establishment and maintenance of gestation in all mammalian species so far studied in detail⁴, and we describe here the production and characterization of an anti-progesterone monoclonal antibody which blocks the establishment of pregnancy in mice.

Adult male BALB/c mice from a colony maintained at this Institute were immunized against progesterone-11 α -succinyl-bovine serum albumin (BSA) conjugate (molar ratio of steroid to protein, 26:1) prepared using carbodiimide as the coupling agent. The spleen cells from these animals were fused with the mouse myeloma cell line NS1^{5,6}. Immediately after fusion the cells were cultured in selection medium containing hypoxanthine-aminopterin-thymidine (HAT) and subsequently HT⁵, and the supernatants were screened by a binding assay (see Table 1) for the presence of progesterone-binding antibodies. Vigorous growth of hybrids was observed but the supernatant of only one well displayed marked anti-progesterone activity. Of 14 subcultures established from this well, four remained active and stable over a 5 month period (AA1, BA2, 2A3, AB1). After cloning the hybrid AA1 on soft agar⁷, one colony was selected for further study (III A4.1) and was recloned. Seventeen subcultures grew well and anti-progesterone antibody was detected by a high binding activity in the supernatants of all 17 wells. This, together with later evidence, suggests that III A4.1 is a clone. Ascites fluid formed after cells of clone III A4.1 were injected into mice was found to contain a high concentration of anti-progesterone monoclonal antibody (up to 15 mg ml⁻¹) by immunoelectrophoresis and analytical ultracentrifugation. It was also shown to possess precipitating activity against steroid-BSA conjugate, but not against BSA alone. Specific antisera showed it to be of mouse IgG1 sub-class. The results of similar tests on ascites fluid prepared with cells of subculture AA1 were identical. When tested by the binding assay (see Table 1), 50% binding of radioligand was obtained at dilutions of ascites fluid ranging from 1:3,000 to 1:43,000. Anti-progesterone antibody (AA1, ascites fluid) had an association constant of 0.4×10^9 M⁻¹ (Scatchard analysis).

The specificity of binding in ascites fluid (AA1 and III A4.1) was compared with that obtained for an antiserum (provided by Dr A. P. F. Flint) raised against the same antigen by the conventional procedure in a sheep. The anti-progesterone monoclonal antibody was less specific than the average of the polyclonal antibodies in antiserum 18.2 (Table 1) as noted by others⁸. However, the high concentration of clonal IgG available in the ascites fluid preparation, its relatively low contamination with other proteins and the preservation of clone III A4.1 in liquid nitrogen provided a long-term reproducible source of a suitable reagent for testing anti-fertility properties.

The anti-implantation properties of the monoclonal antibody (clone III A4) were tested in BALB/c mature virgin mice. Five

daily injections of ascites fluid (15 μ l) blocked implantation in all animals, whether given intraperitoneally (i.p.) or intravenously (Table 2: route). To determine the time at which the monoclonal antibody blocked pregnancy most effectively, mature virgin BALB/c mice were given a single i.p. injection at 32 and 109–130 h post-coitum (p.c.). Monoclonal antibody blocked implantation in all 13 mice treated at 32 h ($P < 0.001$, Table 2: time) and in a further four mice treated at 65 h p.c. (Table 3). Pregnancy was also blocked in 9 of 11 mice treated 109–130 h p.c. but implantation occurred and pregnancy appeared to progress normally in two animals (Table 2: time). Control ascites fluid from mouse myeloma McPc 1748 had no effect on pregnancy when injected at 32 h p.c., but when given at later stages (109–130 h) reduced fertility (Table 2: time).

In mice receiving five daily injections, the mean concentration of plasma progesterone was higher in the experimental than the control group of animals on day 10 p.c., but the difference was not statistically significant. The high concentration was associated with a marked increase in the amount of ³H-progesterone bound by plasma *in vitro* ($P < 0.001$, Table 3). The number of

Table 1 Cross-reactivity of anti-progesterone monoclonal antibody and anti-progesterone polyclonal antiserum

	% Cross-reaction	
	Monoclonal antibody III A4.1	Polyclonal antiserum 18.2
Progesterone	100	100
11 α -Hydroxyprogesterone	310	44
Aetiocholanolone	273	<2
5 β -Pregnanedione	82	8
5 α -Pregnanedione	58	12
11 β -Hydroxyprogesterone	26	14
16 α -Hydroxyprogesterone	23	4
5 β -Androstenedione	8	<2
Deoxycorticosterone	<2	4
Cortisol	<2	<2
20 α -Hydroxyprogesterone	<2	<2
17 α -Hydroxyprogesterone	<2	<2
Pregnenolone	<2	<2
17 α -Hydroxypregnenolone	<2	<2
Androstenedione	<2	<2
6 β -Hydroxyandrostenedione	<2	<2
Testosterone	<2	<2
5 α -Dihydrotestosterone	<2	>2
5 α -Pregnane-3 β ,20 β -diol	<2	<2
5 β -Pregnane-3 α ,20 β -diol	<2	<2
5 α -Pregnane-3 α ,20 α -diol	<2	<2
Androsterone	<2	<2
5 α -Androstenedione	<2	<2
3-Epiandrosterone	<2	<2
Oestrone	<2	<2
Oestradiol-17 β	<2	<2
Oestradiol-17 α	<2	<2
Oestriol	<2	<2
16-Epiestriol	<2	<2

Cross-reactivity was determined using monoclonal antibody III A4.1 and polyclonal antiserum 18.2 at a dilution in phosphate buffer of 1:15,500 and 1:1,400 respectively. At this dilution each preparation bound ~50% of the radioligand used in the binding assay. A high concentration of monoclonal antibody was obtained by the injection of cells of clone III A4.1 into BALB/c mice primed with pristane (2,6,10,14-tetramethylpentadecane) 14 days before injection i.p. of $\sim 7 \times 10^6$ cells. The binding assay consisted of 100 μ l supernatant incubated overnight at doubling dilutions with 100 μ l [³H]progesterone (89 Ci mmol⁻¹; 20,000 d.p.m. per 100 μ l phosphate buffer; Amersham). For screening purposes, supernatants were diluted 1:1 (v/v) with phosphate buffer. Unbound radioligand was absorbed by the addition of 200 μ l dextran-coated charcoal, and 20 min later charcoal-bound radioligand was separated from antibody-bound radioligand by centrifugation. Radioactivity in the supernatants indicative of antibody binding was measured by liquid scintillation spectrometry¹⁵. The nonspecific binding of the assay (culture medium with no antibody) was $5.2 \pm 0.8\%$ (mean $\pm$ s.e.m.) of the added radioligand. Competitive binding studies were carried out using increasing amounts of the compounds listed (25–25,000 pg in 25 μ l buffer) and a constant amount of ³H-progesterone (100 μ l buffer containing 20,000 d.p.m.). Incubation conditions and the method of separation of antibody-bound and unbound radioactivity were identical to those used in the binding assay. Cross-reactivity was calculated as the amount of progesterone required to reduce the amount of radioligand bound by one-half, divided by the amount of competing steroid that produced a similar displacement ($\times 100$)¹⁶.

Table 2 Effect of the route and time of administration (h.p.c.) of anti-progesterone monoclonal antibody on blocking of pregnancy in mice

Group	No. pregnant/ no. treated	Total no. of implantations
Route: five daily injections		
Intraperitoneal Experimental	0/6*	0
Control	4/6	20
Intravenous Experimental	0/6*	0
Control	4/6	39
Time: single injection		
32 h p.c. Experimental	0/13*	0
Control	13/14	109
109–130 h p.c. Experimental	2/11	20
Control	7/12	75

Route: BALB/c mice were injected daily at ~10.00 h on days 1, 2, 3, 4 and 5 (day 1 = day of copulation plug; coitus ~02.00 h). Injections consisted of 200 µl 0.9% NaCl containing 15 µl ascites fluid (cloned hybrid III A4.1, experimental), 15 µl ascites fluid (mouse myeloma McPc 1748, control) or 15 µl non-immune mouse serum. Ascites fluid from the cloned hybrid III A4.1 diluted 1:42,000 bound 50% of radioligand when incubated with ³H-progesterone (~20,000 d.p.m.). Ascites fluid from McPc 1748 or non-immune serum showed no detectable binding of ³H-progesterone. Autopsy was performed on day 10 p.c. Time: BALB/c mice were given a single injection i.p. at the times shown (0 h = estimated time of coitus, ~02.00 h of the morning when a copulation plug was found, day 1). Injections consisted of 200 µl 0.9% NaCl containing 15 µl ascites fluid (cloned hybrid III A4.1, experimental) or 15 µl ascites fluid (mouse myeloma McPc 1748, control, see above). Autopsy was performed on days 11–15 p.c.

* Difference between experimental and control group was significant ($P < 0.001$) when tested after the calculation of exact significance probabilities using the randomization procedure first proposed by Fisher¹⁷.

corpora lutea counted in serial sections of ovaries removed at autopsy was similar in experimental (10.0 ± 0.7 , mean $\pm$ s.e.m.) and control (9.0 ± 2.0) groups, though the corpora lutea were frequently small and regressed in mice treated with anti-progesterone antibody. Thus the high concentration of progesterone in peripheral plasma was probably due to the retention of antibody-bound steroid in circulation, and the decline of luteal function to a lack of pituitary and/or placental luteotropin arising from the failure of implantation.

Plasma progesterone levels in mice treated with a single dose of anti-progesterone antibody at 32 or 65 h p.c. and killed on day 11–15 p.c. were less than one-half those found in mice treated daily for 5 days, presumably due to the further decline of luteal secretion of progesterone. The mean plasma progesterone concentration in mice injected at 109–130 h p.c. was similar to that in control animals; in two animals that remained pregnant the values were high (64 and 94 ng ml⁻¹) whereas in a further two mice which were not pregnant values were low (2 and 14 ng ml⁻¹). Circulating monoclonal antibody was still present at autopsy in mice injected only once with cross-reacting IgG as

judged by the greater amount of radioligand bound by plasma *in vitro* ($47.3 \pm 2.3\%$) compared with values obtained in control mice ($15.5 \pm 1.8\%$, $P < 0.001$, Table 3). The binding observed in plasma from control animals was attributed to corticosteroid-binding globulin⁹ as when plasma was heated at 60 °C for 20 min the value was reduced to levels similar to the nonspecific binding values of the assay, whereas in monoclonal-treated animals it was only reduced to 36% due to the presence of anti-progesterone IgG (Table 3).

The results show that anti-progesterone monoclonal antibody given as single or repeated injections during preimplantation will prevent implantation in mice. It was estimated that at equilibrium, anti-progesterone antibody reduced the concentration of readily available progesterone in circulation by >95% during the critical preimplantation stage, when a precise hormonal sequence of progesterone and oestrogens is normally required to sensitize the endometrium for implantation¹⁰ which begins at ~90 h p.c. The finding that a single injection of monoclonal anti-progesterone IgG (200 µg; 10 mg per kg) prevented pregnancy in all 17 mice treated between 32 and 65 h p.c. and that its effect was slightly reduced when it was administered later, may be associated with the limited capacity of antibody to reduce circulating progesterone levels in the face of increasing luteal secretion^{11,12}. The results obtained with this monoclonal preparation in mice showed a substantial improvement compared with those obtained with a polyclonal antiserum in rats in which anti-progesterone antiserum (10 mg; 50 mg per kg) delayed implantation by at least 24 h but blocked pregnancy in only 6 of 14 treated animals¹³.

The mechanism by which this anti-progesterone monoclonal antibody reduced fertility in mice probably differs according to the time of treatment. When injected at 32 h p.c. transport along the Fallopian tubes may have been affected, whereas when injected 109–130 h p.c. the process of implantation itself seemed to be inhibited. Ligand specificity of the monoclonal anti-progesterone antibody used in this study was relatively inferior to that of a conventional antiserum, certain steroids structurally dissimilar to progesterone (such as aetiocholanolone) competing successfully with progesterone for the monoclonal binding site. Whether or not these cross-reactivities are an essential feature of the anti-implantation efficacy of this antibody remains to be established, but products of suitably selected anti-progesterone clones should prove invaluable for further studies of the regulation of fertility.

We thank Professor E. C. Amoroso, Drs A. P. F. Flint, J. C. Howard and D. E. Walters for help and valuable discussions, and Mrs E. Tredget, Miss M. Hamon, Miss P. Hansford and Mr I. R. Fleet for technical assistance.

Note added in proof: It has been demonstrated that an anti-progesterone monoclonal antibody can be prepared with high specificity¹⁴.

Table 3 Effect of anti-progesterone monoclonal antibody on progesterone levels and binding of progesterone in plasma during pregnancy

Treatment	Group	No. pregnant/ no. treated	Plasma conc. (ng ml ⁻¹)	Progesterone	
				Before heating	% Bound After heating
Five daily injections	Experimental	0/6	35.7 $\pm$ 12.6 (6)	54 $\pm$ 9* (6)	ND
	Control	4/6	11.1 $\pm$ 3.8 (4)	10 $\pm$ 4 (4)	ND
One injection, 32 h p.c.	Experimental	0/4	16.5 $\pm$ 4.1*	45 $\pm$ 3* (4)	33 $\pm$ 3* (4)
	Control	4/4	38.8 $\pm$ 2.3 (4)	19 $\pm$ 2 (4)	8 $\pm$ 1 (4)
One injection, 65 h p.c.	Experimental	0/4	8.7 $\pm$ 5.2 (4)	52 $\pm$ 4 (4)	40 $\pm$ 2 (4)
	Control	2/4	31, 32 (2)	14, 15 (2)	6, 8 (2)
One injection, 109–130 h p.c.	Experimental	2/4	43.5 $\pm$ 21.5	45 $\pm$ 6* (4)	ND
	Control	3/4	34.3 $\pm$ 3.3 (3)	13 $\pm$ 2 (3)	7 $\pm$ 1 (3)

Details of treatment are given in Table 2 legend. Blood was collected from mice under ether anaesthesia on days 10–15 and plasma progesterone concentration was measured by radioimmunoassay. Values of % bound were obtained by the addition of a known amount of ³H-progesterone (100 µl phosphate buffer containing 20,000 d.p.m.) to 100 µl plasma diluted 1:10 with phosphate buffer. In selected cases plasma was heated at 60 °C for 20 min to inactivate binding associated with the presence of corticosteroid-binding globulin⁹. Conditions of incubation and the method of preparation of antibody-bound and unbound radioactivity are described in the text. The amount of radioligand bound in plasma was expressed as a percentage of the total amount of ³H-progesterone added. Results are given as mean $\pm$ s.e.m.; numbers of animals are shown in parentheses. ND, not determined.

* Difference between experimental and control group was significant ($P < 0.001$) using Student's unpaired *t*-test.

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Isolation from murine sarcoma cells of novel transforming growth factors potentiated by EGF

A. B. Roberts*, M. A. Anzano*, L. C. Lamb*, J. M. Smith*, C. A. Frolik*, H. Marquardt†, G. J. Todaro† & M. B. Sporn*

Laboratories of *Chemoprevention and †Viral Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205, USA

Polypeptides classified as transforming growth factors (TGFs) have been found in various neoplastic cells and tumour tissues^{1–5}, and most recently in many non-neoplastic tissues⁶. These TGFs are low-molecular-weight (7,000–20,000) acid-stable polypeptides, which induce anchorage-dependent non-neoplastic indicator cells to form progressively growing colonies in soft agar. Certain extracellular TGFs isolated from conditioned medium derived from both rodent sarcoma virus-transformed cells and several human tumour cell lines have been shown to compete with epidermal growth factor (EGF) for membrane receptors^{1,3,4,7}. By direct extraction from Moloney sarcoma virus (MSV)-transformed cells, we have isolated two distinct classes of intracellular TGFs, one of which competes with EGF for receptor binding sites, whereas the other does not. We report here that after purification by HPLC, the colony-forming activity of the TGFs that do not compete with EGF for receptor binding was enhanced 100-fold by the addition of EGF. This ability to enhance the growth-stimulating effects of the TGFs is specific to EGF, as insulin, the insulin-like growth factors, platelet-derived growth factor and nerve growth factor do not show this property. In contrast, the colony-forming activity of TGFs that compete with EGF for receptor binding is not potentiated by EGF.

Direct extraction of MSV-transformed 3T3 cells with acid-ethanol has been shown² to yield TGFs which, when chromatographed on Bio-Gel P-30, seem to behave similarly to the extracellular sarcoma growth factor (SGF) isolated from conditioned medium derived from these cells¹. However, other experiments have shown that the major soft-agar colony-forming activity in the unfractionated acid-ethanol extract of MSV-transformed cells is potentiated by EGF⁸. Using reverse-phase HPLC for further purification, we now demonstrate the heterogeneous nature of these intracellular TGF preparations.

As seen in Fig. 1, the TGF fraction from Bio-Gel P-30 columns (10,000–12,000 molecular weight) can be separated into two major components by HPLC on C₁₈ μ Bondapak

columns using a linear acetonitrile gradient in 0.1% trifluoroacetic acid. One component, which we term the EGF-potentiated TGF, elutes later (38% acetonitrile) than marker EGF (35% acetonitrile), and together with EGF (2 ng ml⁻¹) results in the formation of large colonies (>3,140 μ m²) in soft agar (Fig. 1a). The other component elutes earlier (32% acetonitrile) and induces formation of smaller colonies (>840 μ m²) in soft agar in the absence of added EGF (Fig. 1b). Binding competition studies using ¹²⁵I-labelled EGF showed that only the latter component competes strongly with EGF (Fig. 1c); this is designated 'intracellular SGF', as its behaviour is similar biologically to the extracellular SGF of conditioned medium^{1,7}. In radioimmunoassays using anti-murine EGF antiserum, all these fractions were shown to be free of native EGF (data not shown). The intracellular SGF peak contained 3.7% of the total recovered absorbance at 280 nm and ~80% of the total colony-forming units assayed in the absence of EGF; the EGF-potentiated TGF peak contained 4.2% of the recovered absorbance units and ~90% of the total colony-forming units assayed in the presence of EGF.

The growth-stimulating effect of EGF and each of the two classes of TGFs on NRK cells, clone 49F (ref. 8), was quantified in the soft-agar system described previously². Cells were stained and analysed for colony formation 1 week after seeding in the presence of TGF and/or EGF. Colony size was determined quantitatively using a Zeiss microscope and Videcon television camera coupled to a Bausch and Lomb Omnicon image analysis system and a data terminal. The field of view was displayed in two dimensions and all parameters were derived from the area

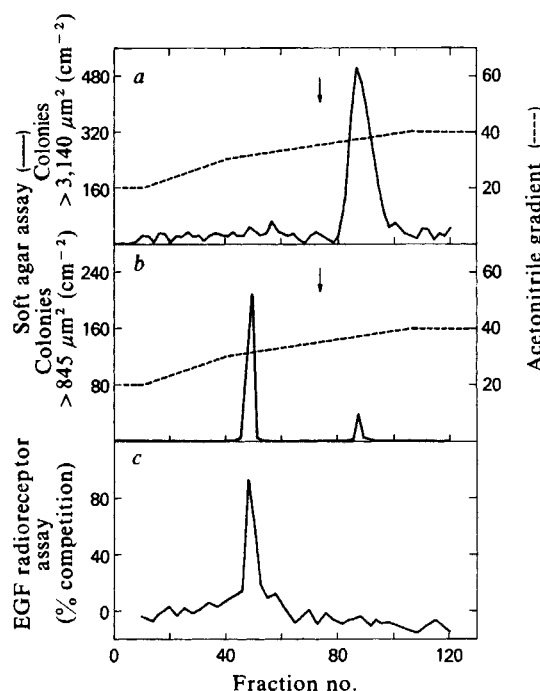


Fig. 1 HPLC purification of TGFs from MSV-transformed 3T3 cells. The acid-ethanol extract of MSV-transformed cells was purified initially on Bio-Gel P-30 in 1 M acetic acid as described elsewhere². EGF was obtained by similar extraction of male mouse salivary glands⁶. The TGF pool (20 mg) was dissolved in 2 ml 0.1% trifluoroacetic acid (TFA), applied to a μ Bondapak C₁₈ column (7.8 $\times$ 300 mm; Waters Associates) and eluted at a flow rate of 0.8 ml min⁻¹ with a gradient of acetonitrile in 0.1% TFA as shown. Aliquots (40 μ l; 5%) were lyophilized and assayed for activity in the soft-agar assay: a, in the presence of 2 ng ml⁻¹ murine EGF; b, without EGF. c, Duplicate aliquots (40 μ l) were assayed for inhibition of ¹²⁵I-EGF-receptor binding in mink lung epithelial cells (CCL 64) as described elsewhere⁷ (3.5 ng ml⁻¹ EGF resulted in 50% competition). Arrow indicates position of elution of marker murine EGF (a, b).

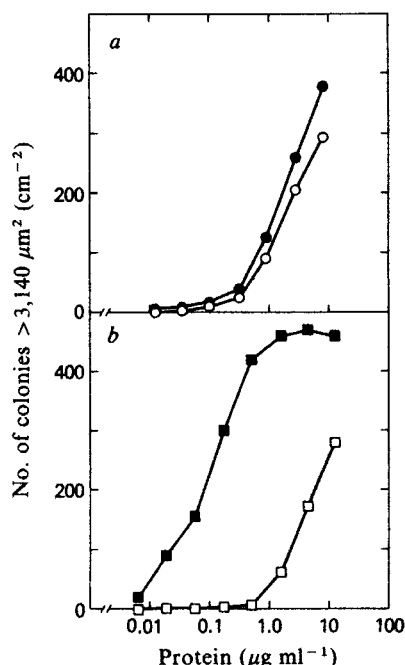


Fig. 2 Effect of EGF on the dose-response curves of the two classes of TGFs. *a*, EGF-independent TGF that competes with EGF for binding (Fig. 1, fractions 49–53) or *b*, EGF-potentiated TGF that does not compete with EGF (Fig. 1, fractions 88–98) was assayed in the absence of EGF (open symbol) or presence of 2 ng ml^{-1} murine EGF (solid symbol). The background level of colonies > 3,140 μm^2 (51 per cm^2) that was observed in response to EGF alone has been subtracted. An identical response was seen for EGF prepared by the method of Savage and Cohen²⁴ and for receptor grade EGF (Collaborative Research).

of the colony cross-section. The system was programmed to size and count colonies > 500 μm^2 and to determine the mean colony size from the data obtained for each plate. The effect of EGF on the dose-response curves of the two classes of TGFs is shown in Fig. 2*a, b*. If the background of colonies formed in response to EGF alone (2 ng ml^{-1}) is subtracted, it is apparent that there is no enhancement by EGF of the intrinsic colony-forming activity of the intracellular SGF (which competes for EGF receptor binding) (Fig. 2*a*). In contrast, the addition of EGF (2 ng ml^{-1}) to the culture medium caused the NRK cells to be ~100-fold more sensitive to the EGF-potentiated class of TGFs, as measured by the number of colonies > 3,140 μm^2 (Fig. 2*b*). In the presence of EGF, soft-agar colony-forming activity was detected in 6 ng ml^{-1} protein from the EGF-potentiated TGF peak after HPLC purification.

The effect on colony size of EGF added to sub-optimal levels of the EGF-potentiated TGF is shown in Fig. 3. EGF alone (2 ng ml^{-1}) induced the NRK cells to form numerous small colonies of mean size 3,300 μm^2 , in agreement with the data of Topp *et al.*⁹, whereas TGF alone (4.6 $\mu\text{g ml}^{-1}$) caused the cells to undergo only a few divisions, to give a mean colony size of 1,800 μm^2 . The simultaneous addition of EGF and TGF resulted in the formation of a large number of colonies of mean colony size 7,700 μm^2 . The numbers of colonies per cm^2 greater than 3,140 μm^2 were 51 and 5, respectively, for EGF and TGF alone, whereas the two factors combined gave 470 colonies, suggesting cooperative effects. The potentiating effect of EGF in the soft-agar assay was optimal at concentrations of 0.4–10 ng ml^{-1} ; higher or lower concentrations had less effect. The response of the NRK cells to EGF alone differed from the response to EGF plus EGF-potentiated TGF; colonies reached a plateau of 2,000–3,000 μm^2 mean colony size in the presence of optimal concentrations of EGF alone, whereas colonies formed in response to the two factors combined reached colony sizes of 13,000–19,000 μm^2 .

Soft-agar assays (data not shown) in which EGF and/or the EGF-potentiated TGF were added sequentially as an overlay 1 week later showed that the order in which EGF or TGF is added is not important for the potentiation of the colony-forming response of the cells, and that the mean colony size attained after sequential addition is not significantly different from that after simultaneous addition of the two factors. In contrast, cells treated initially with EGF alone were refractory to further EGF added 1 week later, and cells treated initially with TGF responded to a further addition of TGF with only a doubling in mean colony size. Other growth factors were investigated for their ability to amplify the anchorage-independent growth response induced by this TGF. Platelet-derived growth factor, nerve growth factor, insulin and the insulin-like growth factors, each tested over a 1,000-fold concentration range, had little effect on the colony-forming response to sub-optimal levels of the EGF-potentiated TGF. By themselves, these factors caused very few cells to undergo even a single division in the soft-agar medium.

The potentiating effect of EGF is specific for a class of TGFs that do not compete with EGF for receptor binding, possibly implicating interaction of two distinct membrane receptors. In model systems, platelet-derived growth factor and pituitary fibroblast growth factor, while not competing for EGF-receptor sites, can induce a transient down-regulation of the EGF receptor, suggesting co-internalization of the receptors for these hormones¹⁰. Phorbol ester tumour promoters^{11,12} and vasopressin¹³ can alter the affinity of EGF receptors, and EGF can alter the response of rat pituitary cells to thyroid hormone and hence alter the hormonal control of growth hormone and prolactin synthesis by these cells¹⁴.

Assuming that anchorage-independent growth correlates with neoplasia^{15,16}, the results reported here suggest a mechanism for some of the observed promoter-like effects ascribed to EGF. EGF can increase viral transformation of cells^{17,18} and promote methylcholanthrene-induced papillomas in mouse skin¹⁹. It can also increase the anchorage-independent growth of adenovirus-transformed rat embryo cells, as measured by increases in both colony size and number²⁰. Each of these effects could be mediated by EGF potentiation of sub-optimal levels of a class of EGF-responsive TGFs resembling those described here.

Results similar to those presented here for the cellular extracts of MSV-transformed mouse cells have also been obtained from both non-neoplastic and neoplastic tissues of man, mouse, chicken and cattle²¹. In every case, the major TGF activity does not compete with EGF for receptor binding but is markedly potentiated by EGF to induce growth of large colonies in soft agar. The presence in these extracts of TGFs that compete with EGF for receptor binding but are not enhanced by EGF in

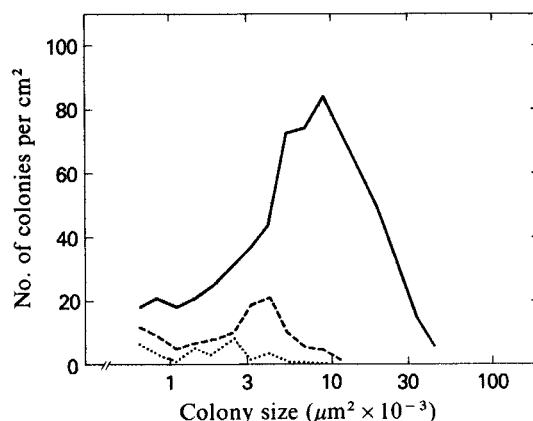


Fig. 3 Size distribution curves of colonies formed in response to EGF and/or sub-optimal concentrations of the EGF-potentiated TGF. The size distribution of colony area, as determined by image analysis, for 4.6 μg TGF (Fig. 2*b*) is represented on a logarithmic scale. $\cdots$, TGF alone (4.6 $\mu\text{g ml}^{-1}$); $—$, TGF at the same concentration in the presence of EGF (2 ng ml^{-1}); and $- - -$, EGF alone (2 ng ml^{-1}) as a control.

the induction of anchorage-independent growth has not yet been conclusively demonstrated. Recent data of Moses *et al.* on TGF production in chemically transformed cells also demonstrate the existence of several TGFs, only one of which competes for binding to the EGF receptor⁵. The physiological significance of the interaction between EGF and this new class of TGFs is unknown, but it is possible that, in addition to a role in malignant transformation, these factors may function in the natural

mechanism of wound repair and in embryogenesis, where both EGF²² and the TGFs²³ have been postulated to play a part.

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Stimulation of tyrosine-specific phosphorylation by platelet-derived growth factor

Bo Ek*, Bengt Westermark†, Åke Wasteson* & Carl-Henrik Heldin*

* Institute of Medical and Physiological Chemistry, Biomedical Center, Uppsala University, Uppsala, Sweden

† The Wallenberg Laboratory, Uppsala University, Uppsala, Sweden

Recent studies have shown that tyrosine-specific protein kinases may be involved in both virus transformation and growth stimulation with polypeptide growth factors. Thus, several *onc* gene products possess such kinase activity¹⁻⁹ and, further, binding of epidermal growth factor (EGF)¹⁰ to A-431 cells stimulates phosphorylation of tyrosine residues of both membrane and cytoplasmic proteins¹¹⁻¹⁶. Platelet-derived growth factor (PDGF), a 30,000 molecular weight (M_r) polypeptide, is the major growth-promoting factor in serum for connective tissue-derived cells and glial cells in culture¹⁷⁻²². High-affinity binding of ¹²⁵I-PDGF to a specific receptor on such cells has recently been demonstrated; thus, human foreskin fibroblasts were found to have some 300,000 PDGF receptors per cell, with an affinity constant of 1 nM (ref. 23). The addition of PDGF to human glial cells and fibroblasts produces various phenotypic changes, similar to those induced by EGF²⁴ and tumour viruses²⁵, which include rapid induction of membrane ruffling, reduction in cell adhesion, change in structure of the actin cytoskeleton and increased mitotic activity (B.W. *et al.*, unpublished data). These considerations suggested that PDGF binding to cellular receptors might similarly stimulate kinase activity. The present report demonstrates such an effect: when incubated with plasma membranes from human fibroblasts or glial cells, PDGF induces the phosphorylation of tyrosine residues of membrane proteins with apparent molecular weights of 175,000 and 130,000.

Membranes were prepared from cultured human foreskin fibroblasts or glial cells as described previously²⁶ and incubated at 0 °C with ³²P-ATP and pure PDGF²¹. After 10 min incubation, the resulting phosphoprotein pattern was analysed by SDS-polyacrylamide gel electrophoresis (PAGE).

As Fig. 1 shows, PDGF stimulated the incorporation of ³²P into membrane proteins of both fibroblasts (Fig. 1a) and glial cells (Fig. 1b), promoting, in particular, the phosphorylation of a 175,000- M_r and a 130,000- M_r protein, in comparison with the

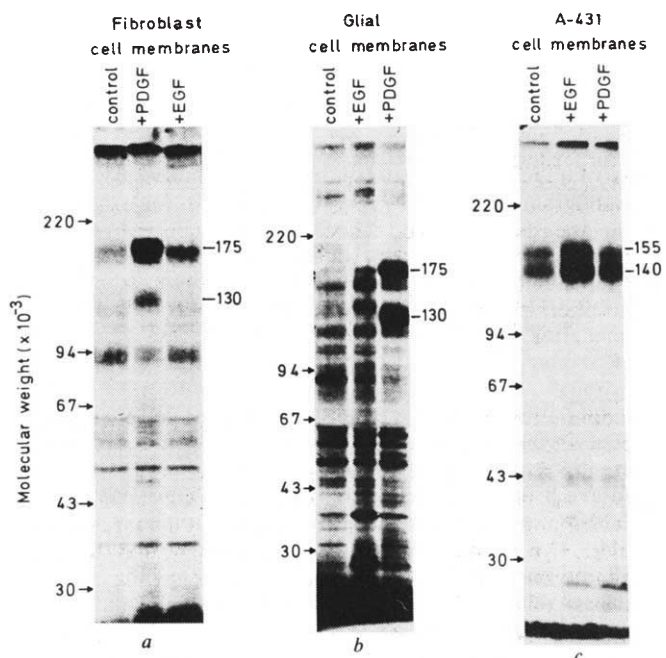


Fig. 1 Effect of PDGF and EGF on the phosphorylation of fibroblast (a), glial (b) and A-431 (c) cell membrane proteins. Cell membranes were prepared from serum-starved cultures of human foreskin fibroblasts (cell line AG 1523, obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, New Jersey), human normal glia-like cells (cell line 1508 CG from our own laboratory) and A-431 cells (obtained from J. E. De Larco, National Cancer Institute, Bethesda, Maryland) according to Thom *et al.*²⁶ as modified in ref. 12, and were stored in aliquots suspended in 20 mM HEPES pH 7.4 at -70 °C for up to 2 months. The phosphorylation reaction was carried out at 0 °C in a final volume of 40 μ l containing 40 μ g (a, c) or 10 μ g (b) of cell membrane protein (determined according to Bradford³¹), 20 mM HEPES pH 7.4, 3 mM MnCl₂, 0.1% bovine serum albumin, 15 μ M (γ -³²P)ATP (33.3 kBq (a, c) or 333 kBq (b)) and 100 ng PDGF (76 nM) or 40 ng EGF (167 nM). PDGF was prepared according to Heldin *et al.*²¹ and EGF was purchased from Collaborative Research. The cell membranes were preincubated with growth factors at 0 °C for 10 min before the phosphorylation reaction was started by adding ³²P-ATP. The reaction was terminated after another 10 min by adding 40 μ l of SDS-sample buffer (80 mM Tris-HCl pH 8.8, 30% w/v sucrose, 3.6% SDS, 10 mM dithiothreitol and 0.01% bromophenol blue) and heating at 95 °C for 3 min. After alkylation with iodoacetamide the samples were subjected to SDS-PAGE as described elsewhere³², using polyacrylamide gradients of 5-10% (a, c) or 3-10% (b) and gel dimensions of 170 $\times$ 1 mm. Gels were run at a constant current of 15 mA. After fixation in 20% 5-sulphosalicylic acid for 1 h, staining with Coomassie blue, destaining and drying under vacuum, the gels were autoradiographed using intensifying screens (DuPont, Lightning Plus) and pre-exposed Kodak X-Omat AR films³³. Exposure was at -70 °C for up to 3 days. The following proteins were used as molecular weight standards: fibronectin (220,000), phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000) and carbonic anhydrase (30,000).

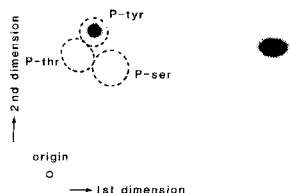


Fig. 2 Phosphoamino acid analysis after PDGF-stimulated phosphorylation. Glial cell membranes (10 μ g) were incubated with 32 P-ATP and PDGF and the incubation mixture analysed by SDS-PAGE (see Fig. 1 legend). The phosphorylated 175,000- M_r component was localized by autoradiography and the corresponding region of the gel excised. The gel piece was hydrolysed under vacuum in 200 μ l 6 M HCl at 110 $^{\circ}$ C for 2 h. After removal of HCl by evaporation under reduced pressure at 37 $^{\circ}$ C, the sample was resuspended in 20 μ l of acetic acid/formic acid/water (78:25:897) containing 2 mg ml $^{-1}$ each of phosphoserine (Sigma), phosphothreonine (Sigma) and phosphotyrosine (synthesized according to ref. 34). Two-dimensional chromatography was performed on cellulose thin layer plates (20 $\times$ 20 cm) as described previously 1 , that is, electrophoresis at pH 1.9 in acetic acid/formic acid/water (78:25:897) at 1 kV for 3 h (first dimension) followed by ascending chromatography in isobutyric acid/0.5 M ammonia (5:3) (second dimension). Autoradiography was performed as described in Fig. 1 legend. Standards were visualized by ninhydrin staining. Broken lines: ninhydrin staining of phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr). The unidentified spot with the highest electrophoretic mobility probably consists of inorganic 32 P formed during the hydrolysis 1 .

control incubation, in which no growth factor was added. Slight effects of PDGF were also seen in other components of the autoradiogram, associated with both increased (for example, a 36,000- M_r protein) and decreased (99,000 M_r) phosphorylation. In a separate experiment to determine the time and dose dependence of the phosphorylation reaction, stimulation of phosphorylation was observed after 1 min at 0 $^{\circ}$ C and was maximal after 5 min; it was detectable in the presence of 8 nM PDGF and was maximal at 40 nM (not shown).

A similar experiment investigated the effect of EGF on phosphorylation of human fibroblast and glial cell membrane proteins. In the presence of EGF, 32 P incorporation increased preferentially in a 170,000- M_r protein of the fibroblast membrane preparation (Fig. 1a) and in both a 170,000- and a 150,000- M_r component of the glial cell membrane preparation (Fig. 1b). This result is clearly distinct from that obtained with PDGF, indicating that different proteins serve as substrates for the kinases stimulated by the two factors. There is evidence that the EGF receptor proper is phosphorylated after stimulation with EGF 13 , but whether PDGF binding induces phosphorylation of the PDGF receptor in an analogous manner remains to be clarified. PDGF seems to be more potent than EGF, producing a higher degree of phosphorylation in fibroblast as well as glial cell membranes. This might be due to the presence of three to five times more PDGF receptors than EGF receptors on both types of cells 23,27 .

The epithelial cell line A-431, which has an unusually high number of EGF receptors 28 , has been shown to lack high-affinity receptors for PDGF 23 . Membranes from this cell line were therefore incubated with 32 P-ATP and PDGF or EGF and the phosphorylation pattern analysed by SDS-PAGE (Fig. 1c). After incubation with PDGF the distribution of phosphoproteins was similar to that of the control carried out in the absence of growth factor. As expected, however, EGF was active in this system, increasing the phosphorylation of proteins in the 140,000–160,000- M_r region. The absence of an appreciable effect of PDGF on membranes from this PDGF receptor-negative cell line in conditions where membranes from PDGF receptor-positive cells did respond to PDGF indicates that the response to PDGF was mediated by binding to the PDGF receptor.

We also investigated the specificity of the PDGF-stimulated kinase activity with regard to the type of phospho-accepting amino acid residue involved. Human glial cell membranes were incubated with 32 P-ATP in the presence or absence of PDGF

and the incubation mixture analysed on SDS-PAGE. The 175,000- M_r component was identified by autoradiography and the corresponding portion of the gel excised. After partial acid hydrolysis the phosphoamino acids were separated on cellulose thin layer plates by electrophoresis at pH 1.9 (first direction) and chromatography in isobutyric acid/0.5 M ammonia (5:3) (second direction) 1 . Figure 2 shows an autoradiogram from this experiment, which indicates that the 175,000- M_r component phosphorylated under the influence of PDGF contained only tyrosine-bound phosphate; no labelled phosphoserine or phosphothreonine was detected. An identical result was obtained with the 175,000- M_r 32 P-labelled product of fibroblast membranes. Analysis of the 130,000- M_r protein also showed the presence of phosphotyrosine, although some phosphothreonine and phosphoserine were detectable (not shown).

In conclusion, PDGF stimulates tyrosine-specific phosphorylation of two membrane components of human fibroblasts and glial cells with apparent M_r s of 175,000 and 130,000, distinct from the 170,000- M_r component phosphorylated in response to EGF. The precise relationship between the individual members of the growing family of tyrosine-specific protein kinases 9,29,30 remains to be elucidated. As they seem to be involved both in the cellular response to growth factors, like EGF and PDGF, and in tumour-virus induced transformation, including the expression of abnormal growth properties, it is reasonable to believe that this type of enzyme participates in the mediation of growth regulatory signals. Therefore, PDGF and EGF, although they bind to separate cell-surface receptors 23 , may function via similar or identical intracellular events, that is, tyrosine-specific phosphorylation of certain growth-controlling proteins. The similarity in some of the phenotypic properties of virus-transformed cells and those of PDGF- or EGF-stimulated cells might then be explained by an overlapping substrate specificity of the respective protein kinases.

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A hypothesis for the initiation of genetic recombination in eukaryotes

Paul Markham & Harold L. K. Whitehouse

Botany School, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK

Initiation of genetic recombination at meiosis in Ascomycete fungi is thought to occur from fixed sites, at least in the majority of events. The simplest way to initiate recombination from fixed sites would be if an endonuclease recognized them and cut one strand of the DNA duplex to allow formation of asymmetric hybrid DNA, as proposed in the Meselson–Radding model of recombination¹. However data from the *Neurospora crassa* *his-3* gene² and the conversion patterns of a mutation in *Schizosaccharomyces pombe*³ and one in *Sordaria brevicollis*⁴, require this idea of initiation to be modified. Here we propose a hypothesis of initiation that will accommodate these experimental observations, which involves the recognition of initiation sites by an endonuclease capable of migrating along a DNA duplex and that cuts in *trans*.

Catcheside and Angel² have described a mutation in *N. crassa* in which the distal quarter of the *his-3* gene with respect to the centromere has been transferred from linkage group I to linkage group VII. On crossing a strain carrying this mutation with strains having a complete *his-3* gene, recombination events were observed in the section of the gene proximal to the break point, but which had been initiated from the distal *cog* site (see Fig. 1). From this they have inferred that recombination events

initiated at the *cog* site⁵, at the distal end of the gene, are capable of migrating down the gene and past the break point, before hybrid DNA formation. These observations require that the mechanism of initiation of recombination from fixed sites allows for the migration of the initiation event before hybrid DNA formation.

The single mutations M26 in the *ade6* gene of *S. pombe*³ and YS17 in the *buff* gene of *S. brevicollis*⁴ are characterized by high conversion frequency, dramatic elevation of levels of interallelic recombination when the alleles are present in a cross and ability to modify recombination polarity. Such behaviour can best be explained if the effect of the mutations has been to introduce fortuitously a site for the initiation of recombination in their respective genes. Both mutations are also converted predominantly to wild type, so that in crosses with wild-type strains, >95% of all aberrant asci produced have three wild-type products of meiosis and one mutant (see Table 1). This observation is clearly at odds with the simple idea of initiation of recombination, which would predict that if M26 and YS17 are initiation sites, they should be recognized by an endonuclease which would cut a single strand of their DNA duplex. This would provide a free single-strand end to invade one of the chromatids of the other parental type, as predicted by the Meselson–Radding model¹, which would result in either correction back to wild type in the invading strand, which would go undetected because it would restore the normal allele ratio, or persistence of the heteroduplex section. Persistence of the uncorrected invading strand would lead to postmeiotic segregation and a three wild-type plus five mutant spored ascus in *S. brevicollis* or an ascus generating one wild-type colony, one sectorized colony and two mutant colonies in *S. pombe*. If correction to mutant occurred in the second strand of the invaded chromatid, then a two wild-type plus six mutant spored ascus would result in *S. brevicollis* or a one wild-type plus three mutant spored ascus in *S.*

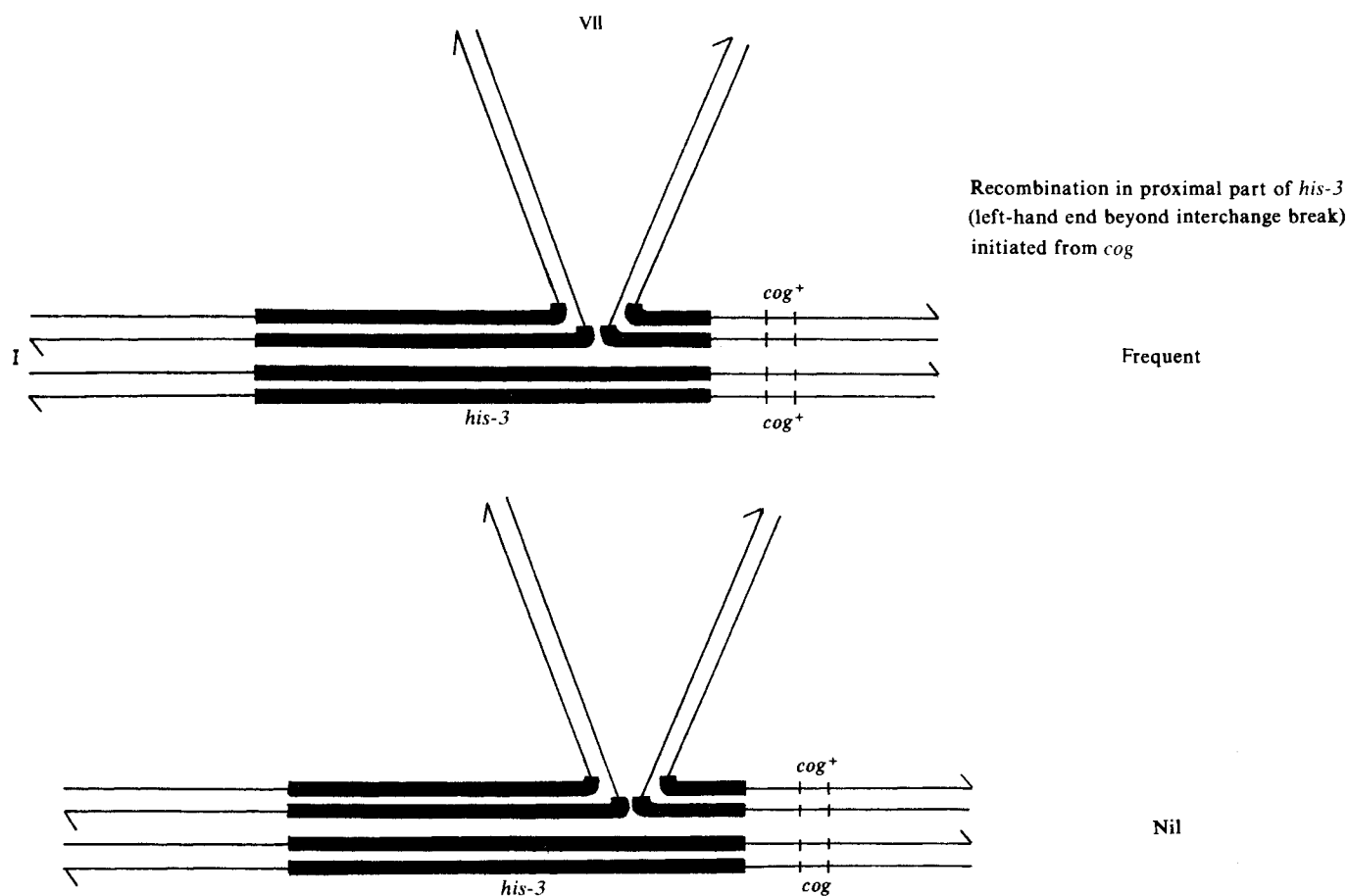


Fig. 1 Diagram illustrating results obtained by Catcheside and Angel² for recombination in the *his-3* gene (thick lines) of *N. crassa* when heterozygous for a *his-3* structural mutant. Not drawn to scale. I and VII indicate the linkage groups.

pombe. This mechanism cannot explain the observed six wild-type plus two mutant spored asci in *S. brevicollis* and three wild-type plus one mutant spored asci in *S. pombe*. This pattern of behaviour is also observed in the *his-3* gene of *N. crassa*. When only one parental strain in a cross carries the more active form of the *cog* site (*cog*⁺), the other carrying a much less active form (*cog*), then alleles of the *his-3* gene present on the molecule with the *cog*⁺ site are preferentially converted to wild type with respect to alleles present on chromatids of the other parental type (*cog*), regardless of the normal gene polarity⁵ (see Table 1). This therefore requires that the DNA strand carrying the initiation site becomes the recipient of hybrid DNA and not the donor.

To accommodate all these observations, we suggest that initiation of recombination from fixed sites occurs by the following mechanism (see Fig. 2). It is assumed that the two homologous DNA molecules, one from each parent, are closely paired before nuclease action. An enzyme complex with endonuclease activity recognizes the fixed site (Fig. 2a) and either cuts a single strand of a chromatid of the other parental type (Fig. 2b), or migrates along the duplex that it has recognized before cutting in *trans* at some random position removed from the initiation site (Fig. 2c). This provides the single-strand end capable of producing hybrid DNA as described by the Meselson–Radding model¹. This hypothesis clearly allows for the migratory capacity necessary to explain the results of Catcheside and Angel² and also explains the results obtained with M26, YS17 and *cog*⁺ by allowing for the cutting of a DNA strand of different parental origin from the initiation site. Catcheside and Angel² originally explained their observations by postulating that the nick or gap introduced at the initiation site was capable of being moved along the DNA strand by combined exonuclease and polymerase activity before giving rise to hybrid DNA. The migration of the initiating enzyme complex and the ability of a gap or nick to be moved along the DNA strand are not, of course, mutually exclusive, so it is useful to examine the idea of nick or gap movement in the light of our

hypothesis. Catcheside and Angel² found that the presence of a fully active (*cog*⁺) site in only one of the *N. crassa* parental strains in a cross involving the *his-3* interchange mutation affected recombination in the part of the gene beyond the break point. Recombination initiated from *cog*⁺ was observed in this region only when *cog*⁺ was in the chromosome carrying the complete *his-3* gene and not when it was in the chromosome carrying the interchange (see Fig. 1). This suggests that if the endonuclease does cut in *trans* then either the nick or gap cannot move along the DNA strand or if it does so it is not recombinogenic, and thus any gap or nick introduced into a complete *his-3* chromatid by an endonuclease recognizing a *cog*⁺ site in a chromatid carrying the interchange cannot result in recombination beyond the interchange point.

The capacity of enzyme systems to carry out the functions required by this hypothesis has been demonstrated in different but closely related systems. Enzyme migration from a recognition site before acting has been postulated for the type I restriction endonucleases of *Escherichia coli* B and *E. coli* K-12^{6–12}. A protein in the *recBC* recombination pathway of *E. coli* is believed to recognize a particular nucleotide sequence^{13,14}. To account for the ability of this enzyme to promote recombination at a distance from such a hot spot, it has been suggested that the enzyme usually slides away from its binding site before nicking the DNA¹⁵. The inability of an intervening non-homologous region to lower the recombination frequency showed that the movement from site of recognition to site of action could not involve the second DNA molecule. Diffusion along the DNA has also been proposed for deoxyribonuclease I of *Ustilago maydis*¹⁶. Cutting in *trans* as a feature of recombination was proposed by Ross and Howard-Flanders^{17,18} for repair in *E. coli* of DNA containing cross-links as a result of exposure to psoralen and 360 nm light. An investigation of recombination between phage λ DNA damaged in this way and undamaged prophage λ , showed that the damaged DNA led to cutting in *trans* of the undamaged homologous DNA. Homology of nucleotide sequence between the two

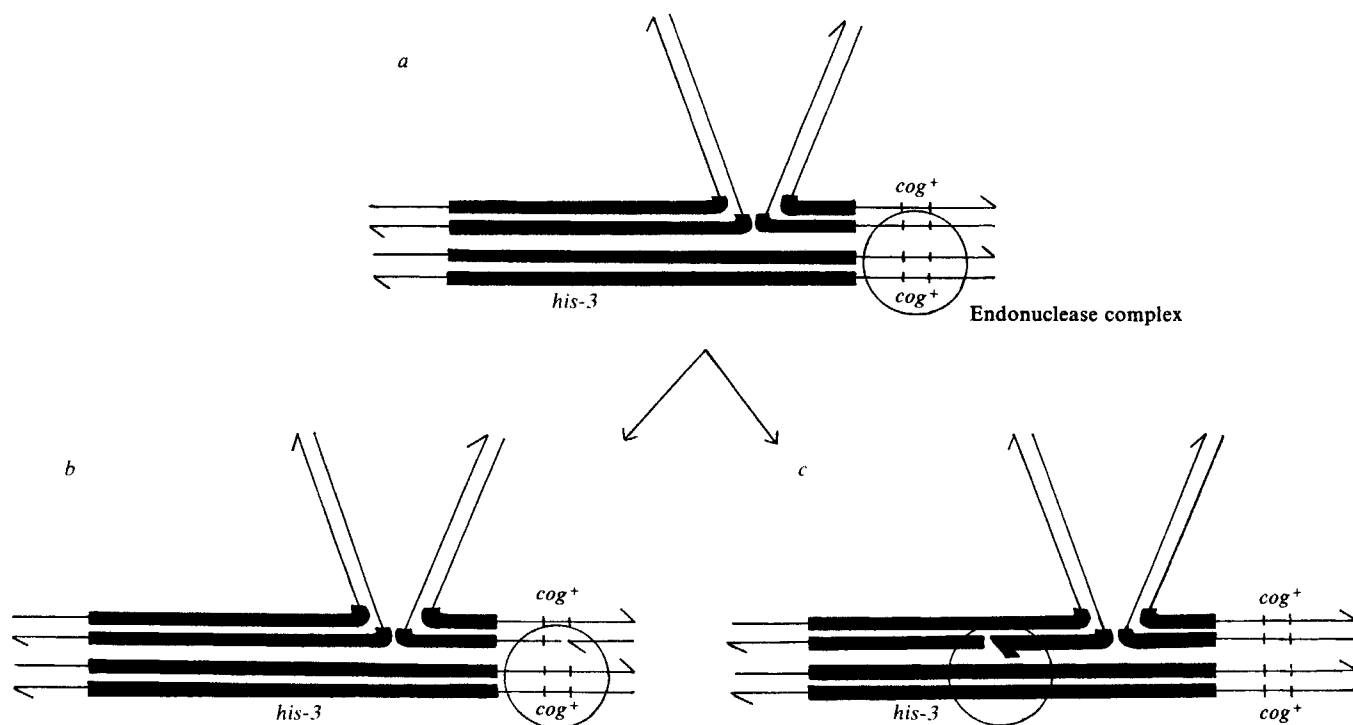


Fig. 2 Diagram illustrating the hypothesis for initiation of recombination. *a*, The endonuclease complex recognizes and binds to the initiation site. Then, either *b*, the endonuclease complex, while still bound to the initiation site, cuts in *trans* one strand of the second chromatid, or *c*, the endonuclease complex migrates along the chromatid which it originally recognized and cuts in *trans* one strand of the second chromatid at a position removed from the initiation site. In this case the enzyme migration leads to a cut being made beyond the interchange break in the second chromatid. After either of these alternative events, invasion of the unbroken chromatid by the free end of the cut strand may lead to hybrid DNA formation. The *his-3* region of *N. crassa* with the interchange break in one chromatid, as illustrated in Fig. 1, is used here to demonstrate the two major features of the hypothesis. The thick lines denote the *his-3* gene. Diagram not drawn to scale.

Table 1 Data from fungal crosses heterozygous for recombination initiation sites

Species and ref.	Parental genotype	Favoured recombinant genotype
<i>Schizosaccharomyces pombe</i> ³	+ M26	+
		+
		+
		M26
		M26
<i>Sordaria brevicollis</i> ⁴	+ YS17	+
		+
		+
		+
		YS17
<i>Neurospora crassa</i> ⁵	M his-3 ^a cog ⁺ N m his-3 ^b cog ⁻ n	M his-3 ⁺ cog ⁺ N
		M his-3 ⁺ cog ⁺ N

M/m and N/n are flanking markers. +, Wild type.

molecules was necessary for the cutting, which required the host *recA* gene function. Subsequently cutting *in trans* was demonstrated *in vitro* using damaged and undamaged molecules of phage ϕ X174 DNA (replicative form I) and crude *E. coli* extracts¹⁹.

Enzyme migration along the DNA from an initiation site, before hybrid DNA formation, offers an explanation for several features of eukaryotic recombination. First, it could account for the occurrence of crossing-over between the sites of alleles without either site apparently being included in hybrid DNA. Such crossing-over is frequent in some genes²⁰ but to account for the polarity often observed in recombination it is necessary to postulate initiation of recombination from fixed sites which appear to be outside genes, such as *cog*. Enzyme migration offers a solution to this paradox by allowing initiation from outside a gene to become manifest only within it on some occasions. Second, there is the mutant specificity of crossing-over between alleles (mutants 137 and 277 in series 46 of *Ascombolus immersus*²⁰ and mutants 4 and 4b at the *g* locus in *Sordaria fimicola*²¹⁻²³). As there is no hybrid DNA at either mutant site with such crossing-over, it was not obvious how the mutant specificity came about. If a mutant had a molecular structure that interfered with heteroduplex formation, selection for recombinants might imply selection for meiotic cells in which enzyme migration had moved hybrid DNA formation beyond the site of the mutation as in the case of the interchange mutation of *his-3* in *N. crassa*. Work with spore colour mutants of *A. immersus* shows that mutations favouring crossing-over with an allele may be deletions. Thus, mutants 1028, 1130 and 49 of series 19 appeared to be deletions because they showed no recombination with two or more closely linked alleles that recombined with one another. They showed frequent crossing-over with more distant alleles²⁰. Such multisite mutations might be expected to interfere with hybrid DNA formation. Third, enzyme migration would allow recombination of distal origin to effect conversion at a proximal site and vice versa, which may have some bearing on apparent cases of polarity reversal.

The Meselson-Radding model of recombination is seen as the most likely consequence of these initiation steps because it best explains data on recombination in eukaryotes. Nevertheless there are alternative mechanisms which could give rise to recombination following the proposed method of initiation.

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Bacterial expression of an enzymatically active protein encoded by RSV *src* gene

J. P. McGrath & A. D. Levinson

Department of Molecular Biology, Genentech, Inc.,
460 Point San Bruno Boulevard, South San Francisco,
California 94080, USA

Genetic evidence indicates that the oncogenic potential of Rous sarcoma virus (RSV) resides in a single viral gene denoted *src*^{1,2}. The product of this gene is a 60,000-molecular weight (*M*) phosphoprotein (pp60^{src})³⁻⁶ which is linked to the plasma membrane of RSV-infected cells⁷⁻⁹ by an interaction at its amino terminus¹⁰. This protein is associated with a protein kinase activity^{5,11} capable of phosphorylating tyrosine residues in acceptor proteins¹²⁻¹⁵. A closely related protein occurs in uninfected cells from a wide variety of vertebrates¹⁶⁻¹⁸ and is presumably encoded by a cellular gene that is alleged to be the progenitor of *src*¹⁶⁻¹⁹. Because detailed enzymological characterization of pp60^{src} has been hampered by a lack of sufficient quantities of purified material, we have produced a bacterial plasmid capable of expressing in *Escherichia coli* a protein closely related to pp60^{src}. We report here the construction of this vector, which directs the synthesis in *E. coli* of large quantities of a protein having the size and immunological properties characteristic of pp60^{src}. In addition, we have demonstrated tyrosine-specific protein kinase activity associated with the bacterially derived protein, an observation which greatly strengthens the claim that the kinase activity previously observed to be associated with pp60^{src} represents a bona fide property of the protein.

The general approach used for the bacterial synthesis of pp60^{src} was to express the protein as a fusion product involving the amino terminus of human growth hormone (HGH) (Fig. 1). HGH has been synthesized directly in *E. coli* under the control of both the *lac* promoter (pHGH107)²⁰ and the *trp* promoter (pHGH207-1)²¹. To minimize the involvement of HGH sequences while preserving the essential control elements of the plasmid (including promoter, ribosome binding site and start codon), we digested cloned pHGH207-1 DNA with *Pvu*II and *Bam*HI and isolated the 4,350-base pair (bp) fragment which contains pBR322 sequences, the *trp* promoter and the first 23 amino acids of HGH²⁰. An *Eco*RI subclone of the Schmidt-Ruppin strain of RSV containing the entire *src* gene (pSRC-RI)²² was cut with *Hae*II and *Bam*HI, and the 2,334-bp fragment (encoding all of pp60^{src} except the first 14 amino acids) was ligated into the *trp* expression plasmid. From the known sequence of HGH²⁰ and pp60^{src} (ref. 22), such a construction was expected to join the HGH and pp60^{src} sequences in the proper reading frame; this was verified by DNA sequence analysis across the HGH-*src* junction (R. Najarian and P. Seeberg, unpublished results). After screening and

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identification of the appropriate recombinant molecules, one such clone (pSRCex16) was tested for the expression of the 60,000- M_r HGH-src protein (p60) in *E. coli*. Because the *trp* promoter is induced by a lack of tryptophan in the medium, it was expected that p60 would be induced in pSRCex16-transformed cultures as depletion of tryptophan occurred. As a specific test of this, cultures of *E. coli* K12 strain 294 were

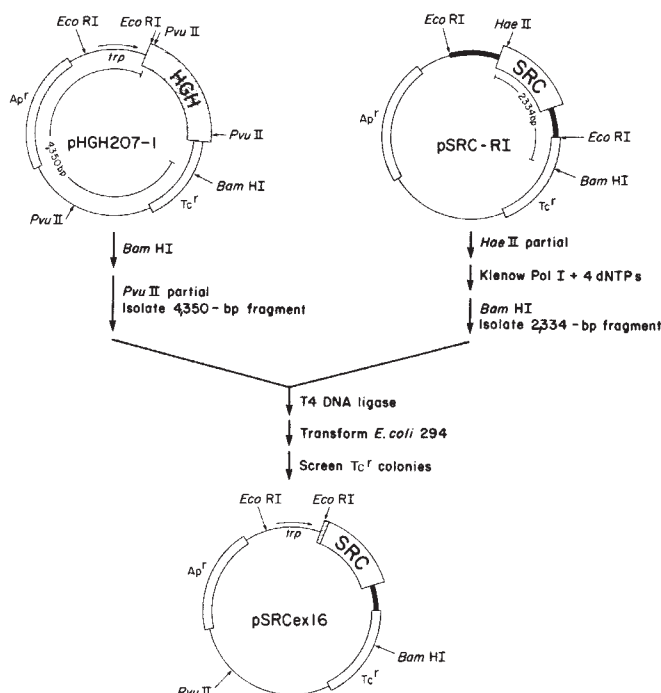


Fig. 1 Construction of a plasmid (pSRCex16) expressing p60. Cloned pHG207-1 DNA²¹ was partially digested with *Pvu*II, then digested to completion with *Bam*HI. The 4,350-bp fragment containing the *trp* promoter, most of pBR322, and the first 69 nucleotides of HGH was isolated from polyacrylamide gels by electroelution³². Plasmid pSRC-RI (ref. 22) was partially digested with *Hae*II, and the 3' single-stranded cohesive termini removed using DNA polymerase I (Klenow fragment) in the presence of four dNTPs. The products were then digested to completion with *Bam*HI, and the 2,334-bp fragment representing 375 base pairs of pBR322 and all but 42 nucleotides of the coding region of pp60^{src} was isolated. The two isolated fragments were ligated to each other using T4 DNA ligase and transformed into *E. coli* 294 (ref. 33). Transformants were selected on LB plates containing 5 μ g ml⁻¹ tetracycline (Tc) and screened for recombinant plasmid DNA by digestion with restriction enzymes. (Enzymes were from either New England Biolabs or BRL and were used according to manufacturer's instructions.)

transformed with either pBR322, pHGH207-1 or pSRCex16 and grown to $A_{550} = 0.25$ or 1.00 in either LB broth (rich medium) or M9 broth (minimal medium). Tryptophan depletion, and consequent activation of the *trp* promoter, have been shown to occur by $A_{550} = 1.0$ in M9 medium^{21,23}. Total cellular extracts were then prepared and the proteins visualized by Coomassie blue staining after electrophoresis on SDS-polyacrylamide gels (Fig. 2). As expected, no overall change in the protein profile occurred in cells transformed with pBR322 regardless of the growth state (Fig. 2a). However, in the pHGH207-1 extract (Fig. 2b), a prominent protein of 22,000- M_r appeared after induction in M9 medium (lanes c, d); this protein was not observed after corresponding times in LB medium, in which tryptophan is abundant (lanes a, b). This protein represents mature HGH when synthesized under *trp* control²¹. Figure 2c demonstrates that in bacteria transformed with pSRCex16, a protein of 60,000- M_r was induced in M9 medium; this corresponds to the size of the protein encoded by *src*²². The protein appeared selectively during late growth phase in M9 medium in bacteria transformed with pSRCex16 (Fig. 2c, lanes c, d), but not in similar cultures grown in rich medium (LB) (lanes a, b), strongly suggesting that it represents the translational product of the *src* gene under the control of the *trp* promoter. We estimate that when induced, this protein represents ~5% of total bacterial protein (170,000 molecules per cell).

To characterize this protein further, we labelled cultures of *E. coli* transformed with either pBR322 or pSRCex16 with ³⁵S-methionine, and prepared lysates for immunoprecipitation. The specificity and efficacy of this procedure have been well documented^{3,5}. Figure 3a illustrates that antiserum reactive against pp60^{src} (TBR serum) specifically precipitated a protein of 60,000 M_r from labelled extracts derived from the 294/pSRCex16 culture (lane d). No corresponding protein was seen with control serum, nor was this protein precipitated when TBR serum was used with an extract derived from a 294/pBR322 culture (lane b). As a further test of specificity, we analysed the bacterially derived 60,000- M_r protein by partial hydrolysis with the V8 protease of *Staphylococcus aureus*, according to the procedure of Cleveland *et al.*²⁴. The digestion products of bacterially produced p60 are virtually identical to those of RSV-encoded pp60 produced in transformed vertebrate cells, when analysed either by ³⁵S-methionine labelling or by Coomassie blue staining (A.D.L. and Hermann Oppermann, unpublished data).

There is much evidence which suggests that the protein kinase activity observed in preparations of pp60^{src} purified from RSV-transformed vertebrate cells represents an inherent property of the protein^{14,15,25}; direct evidence, however, has proved elusive. We therefore assessed the enzymatic activity of bacterially derived p60. We prepared lysates of recombinant *E. coli* strains

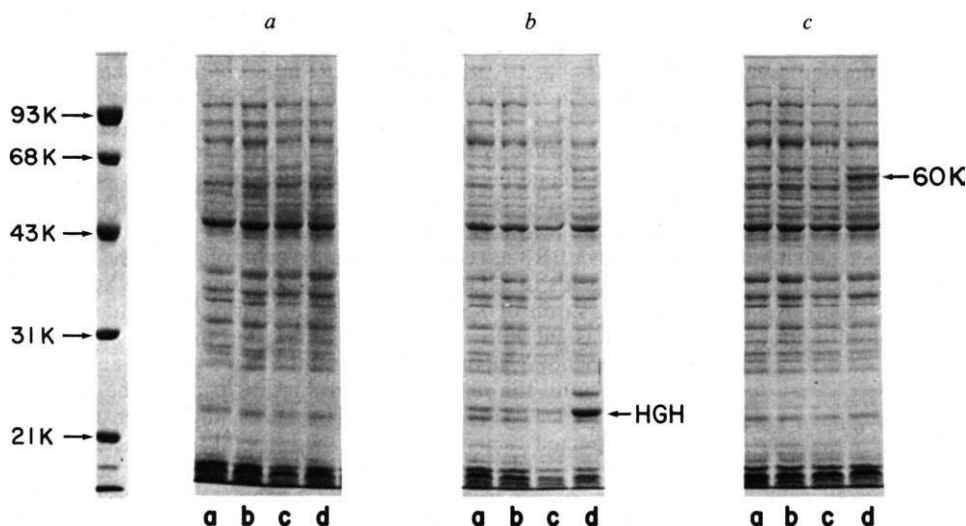
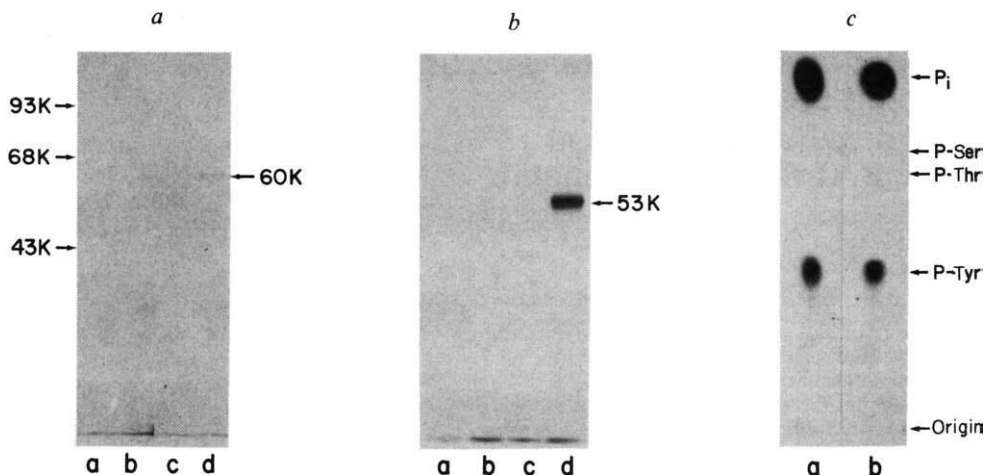


Fig. 2 Analysis of total cellular proteins in *E. coli* transformed with recombinant vectors. *E. coli*, transformed with pBR322 (a), pHGH207-1 (b), or pSRCex16 (c) were grown to $A_{550} = 0.25$ (lanes a, c) or $A_{550} = 1.0$ (lanes b, d) in rich medium (lanes a, b) or minimal medium (lanes c, d) and total cell lysates from 3×10^7 cells were prepared for SDS-gel electrophoresis³⁴ as described elsewhere²⁰. Proteins were visualized by Coomassie blue staining. Molecular weight markers (obtained from BioRad) were electrophoresed in parallel. K represents 1,000 M_r .

Fig. 3 Analysis of *E. coli* strains transformed with recombinant vectors. **a**, Immunoprecipitation of p60 synthesized by *E. coli*. Cultures (2 ml) of *E. coli* strains transformed with either pBR322 (lanes a, b) or pSRCex16 (lanes c, d) were grown to $A_{550} = 1.0$ in M9 medium (10^9 cells) containing $5 \mu\text{g ml}^{-1}$ tetracycline, pelleted, washed, re-pelleted and suspended in 2 ml of M9 supplemented with 0.2% glucose, $1 \mu\text{g ml}^{-1}$ thiamine, $20 \mu\text{g ml}^{-1}$ standard amino acids (except methionine, which was $2 \mu\text{g ml}^{-1}$ and tryptophan which was excluded) $5 \mu\text{g ml}^{-1}$ tetracycline and $20 \mu\text{Ci ml}^{-1}$ ^{35}S -methionine ($1,200 \text{ Ci mmol}^{-1}$; NEN). After incubation at 37°C for 30 min, bacteria were pelleted, resuspended in 0.2 ml 25% sucrose, 50 mM Tris pH 8.0. Lysozyme was added to 1 mg ml^{-1} . After incubation at 4°C for 10 min, the extract was adjusted to 0.5% NP-40, 0.4 M NaCl, 5 mM MgCl_2 and DNase added to $100 \mu\text{g ml}^{-1}$ (Worthington). The extract was incubated at 4°C for 15 min to reduce the viscosity, clarified by centrifugation at $10,000g$ at 4°C for 5 min, and 10% of the supernatant immunoprecipitated with normal baby rabbit serum (lanes a, c) or TBR serum (lanes b, d) and analysed by electrophoresis in polyacrylamide gels as described elsewhere². The autoradiogram was exposed for 3 h. **b**, Immunoprecipitation of a *src*-specific protein kinase activity from recombinant *E. coli* strains. Cultures (1 ml) of *E. coli* strains transformed with either pBR322 (lanes a, b) or pSRCex16 (lanes c, d) were grown to $A_{550} = 1.0$ in M9 medium containing $5 \mu\text{g ml}^{-1}$ tetracycline. The bacteria were pelleted and lysates prepared as in **a**. Samples were immunoprecipitated with normal baby rabbit serum (lanes a, c) or TBR serum (lanes b, d) and IgG kinase assays performed and analysed as described elsewhere¹⁷. **c**, Identification of amino acids phosphorylated by viral pp60^{src} and bacterially derived p60. Immunoglobulin heavy chains phosphorylated by pp60^{src} from RSV-transformed rat cells¹⁵ (lane a) and p60 isolated from *E. coli* (lane b) (see Fig. 2b) were isolated, subjected to partial acid hydrolysis and analysed on cellulose thin-layer plates as described elsewhere¹²; 4,500 c.p.m. from each heavy chain were applied to the origin (marked by an arrow). Autoradiography was performed using a fluorescent screen for 8 h. Markers (P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine) were run as internal standards and visualized by ninhydrin staining. (Phosphoserine and phosphothreonine were from Sigma; phosphotyrosine was provided by H. Oppermann.)



harbouring either pBR322 or the p60 expression plasmid (pSRCex16), and kinase activity capable of phosphorylating anti-pp60^{src} antibody was assayed after purification by immunoprecipitation as described elsewhere⁵. Figure 3b (lane d) demonstrates that such a protein kinase activity is present in bacteria expressing p60. No activity was present in bacteria not synthesizing p60 (lanes a, b), nor was activity observed in either case when normal serum was used (lanes a, c).

Previous evidence¹²⁻¹⁵ has indicated that the kinase activity associated with pp60^{src} phosphorylates acceptor proteins at tyrosine residues rather than at serine and threonine residues, which more typically serve as targets of protein kinase action. To examine the substrate specificity of p60 produced in *E. coli*, we characterized the amino acid on the IgG molecule phosphorylated by bacterially derived preparations of p60. ^{32}P -labelled IgG was isolated and subjected to acid hydrolysis, and the resulting free amino acids separated by TLC¹². Figure 3c reveals that all detectable incorporation of label into antibody occurred at tyrosine residues following reactions using either pp60^{src} derived from RSV-transformed rat cells¹⁵ as a control¹² (lane a), or p60 derived from *E. coli* (lane b). We have attempted to label *E. coli* harbouring the p60 expression plasmid with ^{32}P *in vivo* to identify possible bacterial targets of p60 action, but have so far been unsuccessful. Similarly, we have been unable to detect any 'self' phosphorylation of p60 *in vivo*.

The specificity of the IgG kinase assay has been well documented^{5,11,16,17}, and has provided the basis for the conclusion that pp60^{src} possesses protein kinase activity. However, the possibility that pp60^{src} specifically interacts with and activates a cellular protein kinase responsible for this activity has not been excluded. Our demonstration that bacterially derived p60 also exhibits protein kinase activity addresses this issue, as given the considerable evolutionary divergence between bacteria and vertebrates, and more specifically the lack of any detectable homology between the *src* gene and DNA from *E. coli*¹⁹, we would expect no specific interaction between the avian gene product (p60) and protein kinases of *E. coli*. Furthermore, *E. coli* has been reported to exhibit no²⁶⁻²⁸, or only a very low level²⁹⁻³¹ of protein kinase activity that is specific for phosphorylation at serine and threonine residues^{29,30}. We therefore consider that this demonstration of tyrosine-specific protein kinase activity in a highly purified bacterially derived preparation of p60 lends consider-

able support to the conclusion that this enzymatic activity represents an intrinsic property of the molecule.

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Note added in proof: Since the submission of this letter, similar results have been published by Gilmer and Erikson³⁵.

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Association between a transposed α -globin pseudogene and retrovirus-like elements in the BALB/c mouse genome

Kira Lueders*, Aya Leder†, Philip Leder†‡ & Edward Kuff*

* Laboratory of Biochemistry, National Cancer Institute, and

† Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205, USA

‡ Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

Mice have three functional α -globin genes and two closely related pseudogenes¹⁻⁵. In BALB/c mice, the functional genes are known to be clustered on chromosome 11, but the pseudogenes, designated $\alpha\psi 3$ and $\alpha\psi 4$, are located on chromosomes 15 and 17, respectively⁵. One of the pseudogenes, $\alpha\psi 3$, has precisely lost the intervening sequences characteristic of the functional genes^{3,4}, as if it had been generated from a processed RNA intermediate. It has been suggested that retroviral functions could have been instrumental in the formation of this 'spliceless' pseudogene⁶ and its integration at a novel chromosomal locus⁵. We report here that retrovirus-like elements are located on both sides of the $\alpha\psi 3$ gene.

The genome of *Mus musculus* contains, in addition to endogenous type B and C retroviral genes, 1,000–2,000 copies of retrovirus-like elements related to the mouse intracisternal type A particle (IAP)⁷⁻¹⁰. The IAP-related sequences are associated with many if not all of the mouse chromosomes⁷. We have isolated multiple examples of IAP genes from BALB/c DNA as recombinants in λ phage Charon 4A^{9,10}; a common form is

about 7.3 kilobases (kb) long, colinear with the IAP 35S genomic RNA and bounded by 0.3–0.4 kb of terminal repeated sequences (LTRs). Deleted forms of the IAP genetic units have also been observed^{8,10}.

As IAP genes are abundant and widely distributed throughout the mouse genome, and as integrated retroviruses can have important effects on nearby genetic elements¹¹, we (K.K.L. and E.L.K.) have been systematically searching for proximities between IAP and other known mouse genes. Originally, we found IAP-related sequences associated with 1% of recombinants in a mouse genomic library consisting of ~15 kb inserts in λ phage⁹. Since then, we have tested 98 recombinants selected because they contained an identifiable mouse gene (unrelated to IAP genes). Five of these clones gave positive reactions when the DNAs were spotted on nitrocellulose membranes and hybridized with ³²P-labelled DNA prepared from an isolated IAP gene, MIA14 (refs 9, 10). Among 13 recombinants containing the various α -globin genes and pseudogenes⁵, positive reactions were confined to 2 containing the $\alpha\psi 3$ sequences. As described below, the spatial relationships between IAP and pseudogene regions were then determined by examining heteroduplexes between these clones and two representative recombinants that contain IAP genes in known orientation and position with respect to the λ arms⁹.

Some structural aspects of the recombinants used for heteroduplexing are indicated in Fig. 1a, b. The IAP isolates do not share flanking sequences and have no known topographical relationship to one another. The two $\alpha\psi 3$ -containing clones represent overlapping fragments that span about 22 kb of the mouse genome⁵. In $\alpha\psi 3$ -1, a deletion (Δ) has interrupted the pseudoglobin sequence and brought the truncated gene into juxtaposition with DNA sequences that are located at some distance downstream in the intact genome. In $\alpha\psi 3$ -2, the original relationships of the $\alpha\psi 3$ gene to its flanking sequences have been maintained; this clone shares sequences with $\alpha\psi 3$ -1 in the region of overlap to the left of the pseudogene (see also Fig. 1g).

Representative heteroduplexes are shown in Fig. 1c, d, while

Fig. 1 Heteroduplex mapping of IAP sequences in BALB/c genomic clones containing the pseudo α -globin gene $\alpha\psi 3$. The cloned genomic fragments were derived from mouse embryo DNA by partial *Hae*III digestion and inserted into λ Charon 4A via *Eco*RI linkers. All the inserts are shown in the same orientation with respect to the phage arms, that is, with the left arm towards the left of the figure. The 5'-3' orientations of the genes themselves are indicated by arrows. The λ arms are not shown. The heteroduplexes were prepared and examined as described previously^{9,10}. **a**, Diagrams of two previously mapped recombinants^{9,10} containing 7.3 kb IAP genes in opposite orientations. The IAP sequences are indicated by heavy lines with thickened ends representing the LTRs. The flanking sequences in the two clones (thin lines) are unrelated. **b**, Two recombinants known⁵ to contain $\alpha\psi 3$ gene sequences (filled bars) in the indicated locations. The cloned fragments represent overlapping segments from the same region of the mouse genome⁵; however, in $\alpha\psi 3$ -1, a cloning deletion (Δ) has interrupted the $\alpha\psi 3$ gene (jagged edge) and brought it into continuity with more distant downstreams flanking sequences (light broken line). **c**, Typical heteroduplex between $\lambda\alpha\psi 3$ -1 and λ MIA14. A relaxed SV40 DNA molecule (*) provides a 5.2 kb size standard. **d**, Heteroduplex between $\lambda\alpha\psi 3$ -2 and λ MIA2. **e**, **f**, Semi-diagrammatic representations of the heteroduplexes in **c** and **d**, respectively, showing the known positions of the IAP and $\alpha\psi 3$ sequences on the individual strands. The λ arms are indicated by heavy broken lines. **g**, The two $\alpha\psi 3$ -containing clones, showing the positions and orientations of the IAP sequence elements. In $\lambda\alpha\psi 3$ -1, the presence of the cloning deletion (Δ) is indicated and the right-hand flanking sequences are detached to show that they occupy a position downstream in the intact mouse genome⁵.

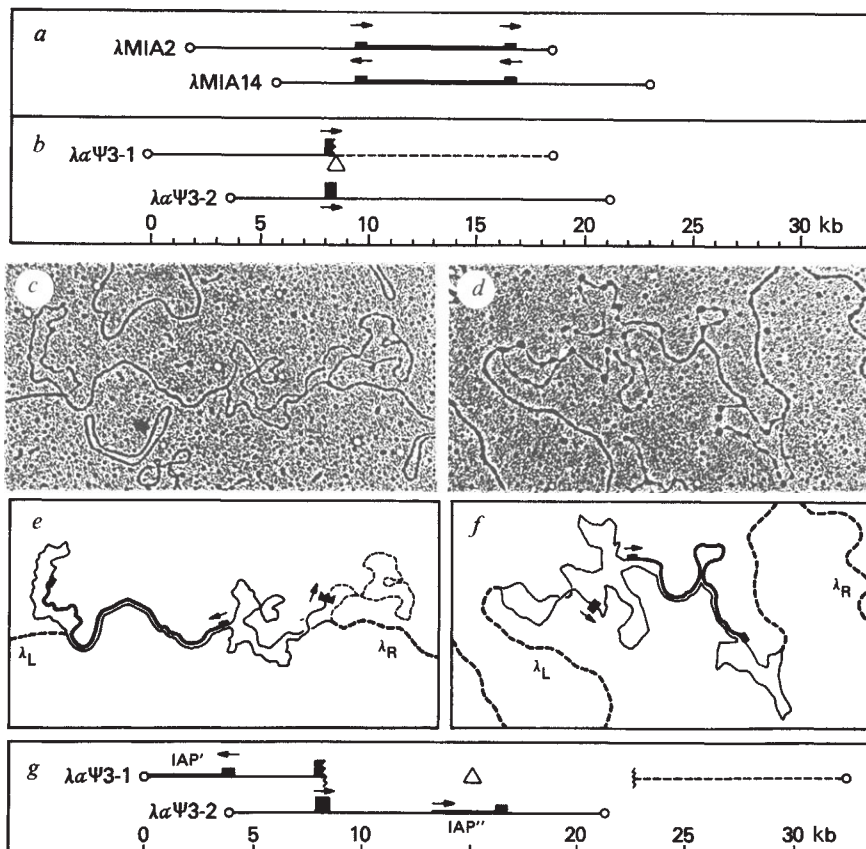


Fig. 1e, f show the individual strands of each heteroduplex, on which we have indicated the known locations of the IAP and pseudoglobin sequences. In Fig. 1c, e, the $\lambda\alpha\psi 3$ -1 strand shows 4 kb of homology with the IAP gene in λ MIA14 before being interrupted at the junction with the λ arm. The IAP and $\alpha\psi 3$ sequences in this clone have opposed 5'-3' orientations. $\lambda\alpha\psi 3$ -2 also has a major region of homology with the IAP gene in λ MIA2 (Fig. 1d, f). The IAP sequences in this clone show two major alterations with respect to the complete IAP gene; one involves the loss of 0.8–1 kb of 5' sequence including the 5' LTR; the other represents the deletion of 3 kb of internal sequence. It is unlikely that these deletions are artefacts produced in cloning because deletions of similar size and position have been seen in several other IAP gene isolates^{8,10}. In fact, three of the other IAP genes found in association with known mouse genes contained deletions (K.K.L. and E.L.K., unpublished observations). Figure 1g summarizes the locations of the IAP and $\alpha\psi 3$ sequences in the $\lambda\alpha\psi 3$ clones. The structures of the clones as deduced from the heteroduplexes were confirmed by restriction enzyme analysis and hybridization with specific probes derived from selected regions of the IAP gene (data not shown).

Figure 2a shows a reconstruction of the genomic region encompassing the $\alpha\psi 3$ gene in BALB/c mice. This putative arrangement, in which the gene is bracketed at a distance of ~4 kb on either side by IAP sequences (IAP' and IAP''), was verified by direct restriction analysis of cellular DNA. The individual $\alpha\psi 3$ -containing clones were first mapped with respect to several restriction endonuclease sites and these sites were positioned on the reconstructed genomic segment (Fig. 2b). The predicted restriction fragments were then sought in digests of BALB/c embryo DNA using hybridization probes (see Fig. 3 legend) derived from the 4.7 kb $\alpha\psi 3$ -containing *EcoRI* fragment shown in Fig. 2b. The results (Fig. 3) were entirely consistent with the proposed gene order. The 11.5 kb *SstI* fragment provided the most direct evidence for this arrangement because it is defined by sites located in the two IAP sequences themselves (Fig. 2). *SstI* fragments of the same size were detected with the $\alpha\psi 3$ probe in the DNAs from several other mouse strains and cell types (livers of AKR/N, C3H, A, C57BL and NIH Swiss mice, BALB/c myeloma and 3T3 fibroblasts), suggesting that the gene order may be a general property of *M. musculus* laboratory strains and is relatively stable in conditions of continued cell division.

Retroviruses are known to acquire and transmit cellular genes^{6,12,13} and Goff *et al.*⁶ have emphasized that such transfer

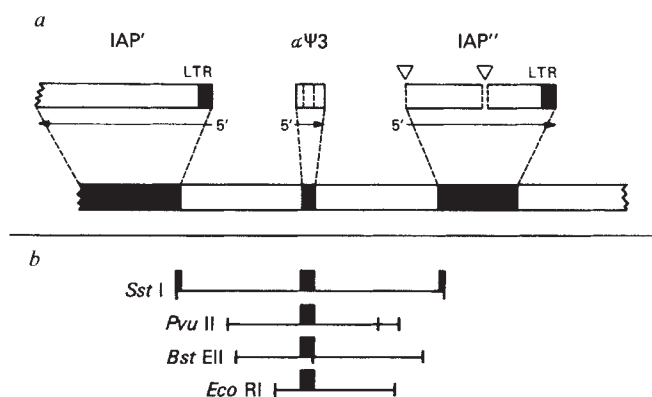


Fig. 2 Putative arrangement of $\alpha\psi 3$ and IAP sequences in the BALB/c genome and some restriction endonuclease fragments predicted from this arrangement. *a*, The continuous bar represents a 23 kb segment of genomic DNA reconstructed from the two individual $\alpha\psi 3$ clones shown in Fig. 1g. The expanded gene sequences at the top show the positions of the IAP LTRs and the two deletions in IAP''. The vertical broken lines in the $\alpha\psi 3$ gene indicate the positions of two intervening sequences found in the closely related functional adult α -globin genes but absent in this pseudogene³. *b*, Putative locations of *SstI*, *PvuII*, *BstEII* and *EcoRI* restriction endonuclease sites in the regions flanking the $\alpha\psi 3$ gene. The individual $\lambda\alpha\psi 3$ clones were first mapped with respect to the enzymes, using probes derived from the 4.7 kb $\alpha\psi 3$ -containing *EcoRI* fragment separately cloned in λ gtWES³. When these sites were then positioned on the reconstructed genomic region, they yielded the indicated set of predicted fragments.

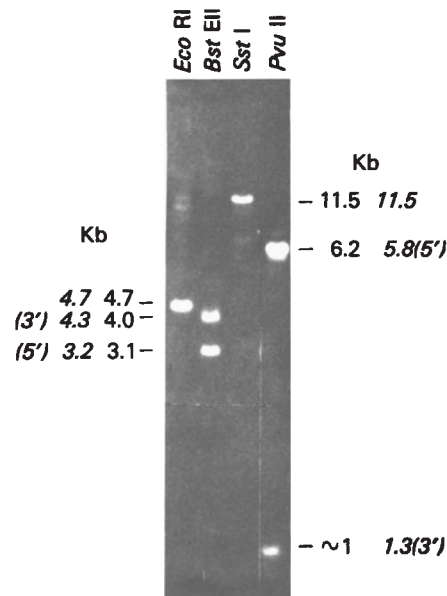


Fig. 3 Comparison of observed genomic restriction endonuclease fragments with those predicted from the sequence arrangement shown in Fig. 2. BALB/c embryo DNA was digested to completion with the indicated enzymes and digests equivalent to 20 μ g of DNA were applied to each lane for electrophoresis in 1.2% agarose gels. After transfer to nitrocellulose membranes the fragments were hybridized sequentially with ³²P-labelled fragments derived by *BstEII* cleavage of the 4.7 kb $\alpha\psi 3$ -containing *EcoRI* fragment (cloned in λ gtWES) shown in Fig. 2b. The blots were autoradiographed after each hybridization step. In this way, the reactive fragments in the *BstEII* and *PvuII* genomic digests were identified in terms of their 5' or 3' position with respect to the $\alpha\psi 3$ gene. The observed sizes of the reactive restriction fragments are shown in upright numerals, the predicted sizes in italics.

can result in the loss of intervening sequences within the acquired gene after one cycle of virus infection. The topographical relationships shown in Fig. 2 lead us to ask whether IAPs could have had a role in generating the $\alpha\psi 3$ gene and/or bringing it to its present location. Because the observed organization of the IAP sequence elements on either side of the $\alpha\psi 3$ gene does not permit us to construct a straightforward model for the events in question, it cannot be ruled out that the observed spatial association is fortuitous. On the other hand, although IAPs are not horizontally infectious, they do have several retrovirus-like properties, and their LTRs have a retroviral type of organization^{10,14} and contain signal sequences appropriate for the initiation and termination of RNA transcription (L. A. Smith and E. L. Kuff, unpublished observations). In addition, IAPs are expressed in pre-implantation embryos^{15–17}, that is, at a developmental stage that would facilitate integration of a recombinant provirus into germ-line DNA.

Perhaps the evolutionary aspects of the situation offer the best hope of resolving this problem. It has been estimated that a functional precursor of the $\alpha 3$ pseudogene may have been generated about 24 Myr ago and eventually become inactive as long ago as 17 Myr¹⁸. Even allowing for major uncertainties inherent in such calculations, these intervals are large with respect to the estimated time of divergence of mouse and rat species from a common ancestral form^{19,20}. IAP-related sequences are found not only in these^{21,22} but in other more distant rodent species such as gerbil and Syrian hamster²³, so that it is likely that IAP genes or their evolutionary precursors were extant at the time the ancestral $\alpha 3$ pseudogene was formed. Comparative observations using gene libraries prepared from other species may clarify the possible role of IAPs in the evolution of the pseudo α -globin gene.

Note added in proof: Evidence has recently been obtained for the presence of $\alpha\psi 3$ genes in the DNAs of other species of *Mus* and of other more distantly related rodents such as rat, gerbil and hamster (R. Taub and A. Leder, unpublished observations).

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Structural alterations in J regions of mouse immunoglobulin λ genes are associated with differential gene expression

Jim Miller, Erik Selsing & Ursula Storb

Department of Microbiology and Immunology, University of Washington, Seattle, Washington 98195, USA

Immunoglobulin λ light chains in the mouse comprise three types: $\lambda 1$, $\lambda 2$ and $\lambda 3$ (refs 1–3 respectively) and are encoded by three constant region (C λ) and two variable region (V λ) genes. The C $\lambda 2$ and C $\lambda 3$ protein sequences differ at only 5 amino acid positions whereas C $\lambda 1$ differs from both by about 30 amino acids. The C $\lambda 1$ and C $\lambda 3$ genes are closely linked⁴, separated by ~3 kilobases (kb) of DNA. The C $\lambda 2$ gene is closely linked to a C $\lambda 4$ gene, but is an unknown distance from the C $\lambda 3$ –C $\lambda 1$ cluster^{4,5}. Although the C $\lambda 4$ gene shares homology with C $\lambda 1$, we show here that it represents a pseudogene. Only two germ-line V genes appear to be expressed in mouse λ chains: V $\lambda 1$ is used in association with either C $\lambda 1$ (ref. 1) or C $\lambda 3$ (ref. 6), while V $\lambda 2$ has only been found with C $\lambda 2$ (ref. 2). Differential gene expression is observed in the λ locus. Among mouse serum antibodies, $\lambda 1$ chains occur 10 times more frequently than $\lambda 2$ (ref. 7) or $\lambda 3$ (ref. 3) chains while $\lambda 4$ chains have not been identified. We have now searched for structural differences between C λ genes which could explain this differential expression. Our nucleotide sequence analysis indicates that C $\lambda 4$ is not expressed due to an inactive RNA splice site. Although no structural defects were found in C $\lambda 2$ or C $\lambda 3$, substitutions in the rearrangement recognition sequences associated with J $\lambda 2$ and J $\lambda 3$ segments, together with nearby competing pseudo recognition sequences, may explain the relatively low levels of C $\lambda 2$ and C $\lambda 3$ gene expression.

Immunoglobulin gene expression requires the recombinational joining of V and C gene segments via a small joining (J) DNA segment^{8–11}. J segments characteristically display V–J joining and RNA splicing recognition sequences separated by a short coding sequence (13 amino acids). We have found J segments 1.2–1.4 kb 5' of each C λ gene (Fig. 1). In each J λ segment, sequences showing homology to V–J joining and RNA splice sites are observed (Fig. 2). In the J $\lambda 1$, J $\lambda 2$ and J $\lambda 3$ genes the coding sequences correspond exactly to the J peptide segments in $\lambda 1$, $\lambda 2$ and $\lambda 3$ light chains respectively (refs 2, 12 and H. Eisen, personal communication).

The J region associated with C $\lambda 4$ was of particular interest because $\lambda 4$ proteins have not been observed among mouse light

chains; comparison of the J $\lambda 4$ sequence with the closely homologous J $\lambda 1$ sequence suggests an explanation for this observation (Fig. 2). Relative to J $\lambda 1$, J $\lambda 4$ has both a deletion of two nucleotides near the beginning of the coding sequence and a nucleotide substitution in a critical position of the RNA splice site. The substitution changes the G–T, found in all 5' intron sequences¹³, to an A–T. Even if this unconventional splice site (a in Fig. 2) were used, the 2-base pair (bp) deletion at the beginning of J $\lambda 4$ would result in a shortened, and possibly nonfunctional, mRNA. A possible alternative splice site for J $\lambda 4$ (b in Fig. 2) results in termination codons in all mRNA reading frames, either in J $\lambda 4$ (Fig. 2) or in C $\lambda 4$ (E.S., J.M., R. Wilson and U.S., in preparation). Apparently, rearranged V $\lambda 2$ –J $\lambda 4$ –C $\lambda 4$ genes would not result in an active mRNA. Thus, C $\lambda 4$ represents a nonfunctional pseudogene.

Although the events leading to the inactivation of the C $\lambda 4$ gene cannot be ordered unambiguously, it is possible that, like J $\kappa 3$ in the κ gene complex^{8,9}, J $\lambda 4$ initially became inactive due to an alteration in its RNA splice site. Subsequent evolutionary drift could have resulted in the 2-bp deletion.

Mouse κ and H chain C genes are associated with four functional and one nonfunctional J gene segment^{8–11}. Known λ protein chains only contain one type of J segment: J $\lambda 1$ for $\lambda 1$, J $\lambda 2$ for $\lambda 2$ and J $\lambda 3$ for $\lambda 3$ (refs 12, 2 and H. Eisen, personal communication, respectively). A corresponding J segment is present 5' of each of the C λ genes (Fig. 1). No additional J segments were found in the regions 250–350 bp 3' of J $\lambda 1$, J $\lambda 2$, J $\lambda 3$ or J $\lambda 4$, that is, at the distance J segments are spaced in κ and H chain genes.

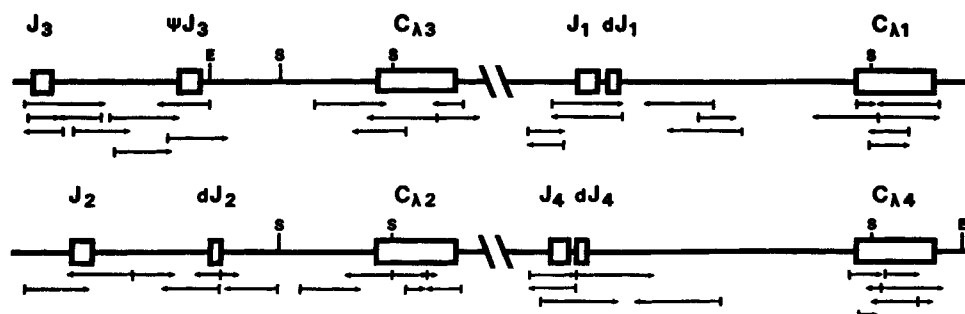
However, there is another J-like region 601 nucleotides 3' from the end of J $\lambda 3$ (see Fig. 1). Identified by sequences homologous to V–J recombination and RNA splice sites, this J also contains a potential coding region of the appropriate length, in which all three possible reading frames are open (Fig. 2). Nevertheless, certain characteristics of J regions are missing. First, the recognition sequence contains a poorly matched heptamer. Second, the nucleotides in the potential coding region have little resemblance to those of known J segments. Furthermore, this resemblance is no greater when these nucleotides are translated into amino acids in any of the three open reading frames. We conclude that this is a pseudo J region (ψ J $\lambda 3$). ψ J $\lambda 3$ differs in structure from the recently detected ψ JH, which contains in-phase termination codons¹⁰.

Although we find a pseudo J sequence associated with C $\lambda 3$, in the analogous position relative to C $\lambda 2$ there is no well matched V–J recombination or RNA splice site (d (downstream) J $\lambda 2$ in Fig. 2). Sequences similar to V–J joining sites are found 117 and 113 bp 3' of J $\lambda 1$ and J $\lambda 4$ (dJ $\lambda 1$ and dJ $\lambda 4$ in Figs 1, 2). It appears possible, therefore, that, like C κ and CH genes, C λ genes once had associated multiple J λ sequences but these have been partially lost during evolution.

Do the primary DNA sequences of λ genes suggest a basis for the differential serum concentrations found for $\lambda 1$, $\lambda 2$ and $\lambda 3$ protein chains? Comparison of C $\lambda 1$ and C $\lambda 3$ is most straightforward because both are associated with V $\lambda 1$ in the respective light chains. As only the very conservative substitutions, Trp–Val in J $\lambda 1$ compared with Phe–Ile in J $\lambda 3$, are found in the third hypervariable region¹⁴ of germ-line $\lambda 1$ and $\lambda 3$ genes (Fig. 2), it seems unlikely that differential expression of $\lambda 1$ and $\lambda 3$ results from different affinities for antigen. In addition, we have found normal RNA splicing and translation termination signal sequences associated with C $\lambda 2$ and C $\lambda 3$ as well as with C $\lambda 1$ (the sequencing strategy for the C λ genes is shown in Fig. 1; data will be presented elsewhere: E.S., J.M., R. Wilson and U.S., in preparation). Thus, the low levels of C $\lambda 2$ and C $\lambda 3$ proteins in mouse serum are not due to any apparent faulty processing signals associated with the C $\lambda 2$ and C $\lambda 3$ exons.

Although preferential association of the C $\lambda 1$ protein region with CH regions cannot be ruled out, structural features in the DNA sequences flanking the J genes could explain the differential gene expression. All J segments associated with mouse κ ^{8,9}, H^{10,11} and λ ¹⁵ genes and human κ genes (P. Hieter, J. Maizel and

Fig. 1 Organization and sequence analysis of the four CA genes. Sequencing strategy of the four CA genes and their corresponding J regions (open boxes). Clones containing either the CA3-CA1 (ref. 4) or the CA2-CA4 (E.S., J.M., R. Wilson and U.S., in preparation) gene clusters were obtained from a BALB/c kidney DNA library. The distance between CA3 and CA1 is 3.2 kb, that between CA2 and CA4 is 3.0 kb. The sequence was determined by the dideoxynucleotide termination method²² as described previously²³ or in conjunction with M13 cloning vectors²⁴. The direction and extent of the sequence determinations are indicated by arrows. The pseudo (ψ) and downstream (d) J regions are described in the text. E, *EcoRI*; S, *SacI*.



P. Leder, personal communication) are preceded 5' by two blocks of conserved 'recognition' sequences presumed to be involved in V-J joining: a nanomer with the consensus sequence GGTTTTGT separated by a spacer of 12 (λ) or 21-24 (κ and H) nucleotides from a heptamer with the consensus sequence CACTGTG. Differences in the J λ 1 and J λ 3 recognition sequences could explain the unequal expression of λ 1 and λ 3 genes, assuming homology with the consensus recognition sequence determines the frequency of V-J joining. The recognition sequences of both J λ 1 and J λ 3 exhibit three mismatches when compared with the consensus sequence (Fig. 2). However, the mismatches in J λ 3 are all clustered at the 3' end of the nanomer whereas those in J λ 1 are distributed over both the nanomer and heptamer. No J κ or JH recognition sequences exhibit multiple mismatches at the 3' end of the nanomer.

Also striking in the J λ 3 recognition sequence is the presence of 'interfering' nanomers. The ψ J λ 3 gene possesses a nanomer which shows greater homology to the consensus sequence than does the J λ 3 nanomer. Also, extraneous nanomers occur in the recognition sequence spacers of both J λ 3 and ψ J λ 3 V-J joining sites (Fig. 2), which are also more homologous with the consensus nanomer than is the functional J λ 3 nanomer. No other JH, J κ , or JA (except J λ 2, see below) exhibits a nanomer sequence in the recognition site spacer. These extra recognition sequences surrounding the J λ 3 region might create substrate competition for putative V-J recombination proteins, which could then interfere with correct recombination to J λ 3, and so decrease the ratio of V λ 1-J λ 3 to V λ 1-J λ 1 rearrangements.

Although it is not known whether λ 2 containing immunoglobulins have less affinity for common antigens than those containing λ 1, the low level of λ 2 expression could result from the same structural defects as in λ 3. For example, there are

deviations from the consensus sequence clustered at the 3' end of the J λ 2 nanomer and an interfering nanomer exists in the recognition site spacer which is more closely matched to the consensus than is the J λ 2 nanomer. In addition, J λ 4 and dJ λ 4 could compete with J λ 2 for V λ 2 rearrangements. As both J λ 4 and dJ λ 4 lack functional RNA splice sites, any rearrangement into these J regions would lead to a nonfunctional transcription unit. These structural features, in and around J λ 2, could interfere with proper V λ 2-J λ 2 joining and result in decreased expression of λ 2.

J λ 1 is also associated with an interfering recognition sequence (dJ λ 1, see Figs 1, 2). This sequence is not as well matched to the consensus as is J λ 1 and, therefore, less likely to interfere with λ 1 expression. Thus, the differential expression of the λ isotypes in normal mouse serum correlates well with the structural integrity of the J region associated with the individual CA genes.

The λ gene family not only shows variation in isotype expression, but as a whole represents only 5% of mouse serum immunoglobulin light chains^{7,16}. Studies of individual immunoglobulin-producing cells have also indicated that the rearrangement frequency is much lower for λ genes than for κ genes^{17,18}. Note that all functional J segments associated with CA genes exhibit a 3'-terminal C or G residue in their recognition sequence nanomers. Because this position is invariably a T in all other J κ and JH segments, it is tempting to speculate that these substitutions might particularly affect the V-J joining rate. However, as several components may differently affect λ and κ genes (such as antigenic selection, specific mechanisms which might rearrange κ genes before λ genes) it would be premature to implicate a specific position in the nanomer sequence. In particular, the ratio of λ and κ rearrangements may only reflect the different numbers of V genes (50-2,000 for κ ^{19,20} compared

	V-J recombination recognition site		RNA splice site
Consensus J	GGTTT [*] TTG [*] T	CACTGTG	AGG [*] TRAG
J λ 1	GTTT [*] TTTGC atgagtctatat	CACAGTG ctgggtgttctggtggaggaacaaactgactgtctcAGG [*] TAAG	TrpValPheGlyGlyGlyThrLysLeuThrValLeuGly
dJ λ 1	GCTTTTGT tgtatacttttc	CIT [*] TCG tattctgttccatacctatacttcacactaggtaaagaatttct	
J λ 2	GGTTT [*] GGG ttgGGTTT [*] AGT	CAITGTG ttatgttttctggcgggtggaacaaaggtcactgtctcAGG [*] TAAG	TyrValPheGlyGlyGlyThrLysValThrValLeuGly
dJ λ 2	GGATCCCGC tgtttcacatttc	CAITGCC agcttctcgttttctcctcaaatggcctagtatatgcaggagatca	
J λ 3	GGTTT [*] AGGG ttgGGTTT [*] CAGT	CACTGTG gttatttttctggcaggtggaacaaaggtcactgtctcAGG [*] TAAG	PheIlePheGlySerGlyThrLysValThrValLeuGly
ψ J λ 3	GGATTTTGC tGTTT [*] GTGttc	CGTIGCC aggttcttttctcctcaaatggcctattgtatgcaggAGG [*] TCAG	ArgPhePhePheLeuLysTrpProIleValCysArgArg GlySerPheSerSerAsnGlyLeuLeuTyrAlaGlyGly ValLeuPheProGlnMetAlaTyrCysMetGlnGluVal
J λ 4	GTTT [*] TTTGC attagactatat	CAGTGTI gggtgttctggagggtggaacagattgactgtctcAGT ^a AG ^b ETGAC	GlyCysSerGluValGluProAspTer GlyValArgArgTrpAsnGlnLeuAspCysProArgTer ValPheGlyGlyGlyThrArgLeuThrValLeuAspGlu
dJ λ 4	GATTTTGT tggataatttct	CATTATG tattctacttcctgctcaaaccttctactctaggtcgaaataatc	

Fig. 2 Comparison of the λ J region DNA segments. The DNA sequences were obtained as described in Fig. 1 legend. Rearrangement recognition sequences and RNA splice sites are in capital letters. Differences from the consensus sequences (see text) are underlined. a And b indicate possible RNA splice sites in J λ 4. dJ λ 1, dJ λ 2, dJ λ 4: recognition sequences located 117, 600 and 113 bp respectively 3' of the beginning of the nanomer preceding J λ 1, J λ 3 and J λ 4. * Indicates positions of the J recognition sequences which are invariant in functional κ and H genes. $\rightarrow$, Only reading frame of J λ 4 which, using RNA splice site a, would also be open in CA4.

with 2 for λ) associated with these families. If recombination occurs equally at λ and κ recognition sites and functional recombinations represent only a portion of these events, then the observed λ/κ rearrangement ratio could merely be a statistical phenomenon. Further investigation will be required to clarify this point.

Our studies suggest that differences in the J regions may have profound effects on the level of immunoglobulin gene expression. In particular, clustering of mismatches at the 3' end of J gene recognition nanomers and competition by interfering recognition sequences may be important. The degree of alteration of the J λ regions is well correlated with their respective frequency of expression. Such alterations may also explain the *cis*-dominant defect that results in very low levels of λ 1 expres-

sion in the SJL mouse strain²¹. In addition, we have found that the J region associated with the C λ 4 gene, whose product has not been found among λ chains, has lost its 3' RNA splice site by a point mutation. Thus the J λ 4-C λ 4 gene represents a pseudogene. Because each C λ gene is associated with only a single J region, unlike κ and H chains⁸⁻¹¹, the inactivation of a J λ region would cause the entire gene to become nonfunctional.

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Nucleotide sequence of a macronuclear gene for actin in *Oxytricha fallax*

Brian P. Kaine* & Brian B. Spear†

Section of Biological Sciences, Department of Biochemistry, Molecular and Cell Biology, Northwestern University, Evanston, Illinois 60201, USA

The hypotrichous ciliated protozoan *Oxytricha fallax* possesses a mechanism for processing its genome into a collection of small, gene-sized, transcriptionally active sections. Macronuclear DNA is arranged as short achromosomal sections, 0.5-22 kilobase pairs (kbp) long, while micronuclear DNA has a typical chromosomal organization^{1,2}. Macronuclear DNA is derived from micronuclear DNA by a process of polytene chromosome fragmentation with a resultant decrease in DNA sequence complexity^{3,4}. The isolation of intact macronuclear DNA sections enables the study of specific structural units of genetic material that occur naturally in the organism. We have previously described⁵ the construction of a recombinant plasmid, pOfACT (1.6), which contains an intact 1.6-kb length of macronuclear DNA (Fig. 1) homologous to a yeast actin gene⁶. We now present the complete nucleotide sequence of the cloned DNA molecule and the derived sequence of the actin protein which it encodes. As the sequence is that of a complete, naturally occurring DNA molecule, it shows all *cis*-active regulatory regions in addition to the coding region. The actin protein encoded differs considerably from other known actins.

The complete nucleotide sequence of the cloned 1.6-kb macronuclear DNA section, determined by the chemical procedure of Maxam and Gilbert⁷, is shown in Fig. 2. The overall length of the 1.6-kb insert from plasmid pOfACT(1.6) is 1,561 bp, measured from the *Pst*I cleavage sites which flank the cloned sequence. This length includes the cleavage sites and the polynucleotide tails which were added enzymatically during construction of the plasmid. Incubation of macronuclear DNA with terminal transferase and cloning in *Escherichia coli* added 24 dG bases to the 5' end and 26 dC bases to the 3' end of the 1.6-kb section.

DNA sequencing has demonstrated an inverted repeat sequence at both ends of the cloned 1.6-kb macronuclear section. This sequence takes the form of a repeating octanucleotide 3'-GGGGTTTT...5'. Subsequent sequencing of total native macronuclear DNA (A. F. Pluta, B.P.K. and B.B.S., in preparation) has confirmed the presence of this terminal sequence on all macronuclear DNA molecules. Similar sequences have been found at the macronuclear termini of other related species^{8,9}. The repeat sequences does not appear at any internal locations in the 1.6-kb clone.

The nucleotide sequence 5' to the actin-coding region but 3' to the terminal repeat is 183 bases long. Several possible promoters for RNA polymerase have been identified in this region. Three sequences beginning at positions 8, 137 and 154 (Fig. 2) resemble the 'Hogness box' sequence, TATAAAA. This sequence (or others closely related to it) has been found in 5' regions flanking numerous eukaryotic genes and is believed to be a transcriptional promoter^{10,11}. Surprisingly, the 'Pribnow box' prokaryotic transcriptional promoter sequence, TATGATG¹², occurs at position 20. If this sequence is indeed a functional promoter, it is possible that *O. fallax* possesses an RNA polymerase similar to that of prokaryotic organisms.

A second group of putative regulatory signals resembles the pentanucleotide CCAAT, which has also been implicated in the control of transcription¹³. These sequences occur at positions -2 (beginning with the last two bases of the terminal repeat on the anti-sense strand), 101, 129 and 152 (Fig. 2). A similar sequence, CCAAA, forms part of the terminal repeats upstream from the actin-coding region. The significance, if any, of this sequence occurring in the repeats is unknown.

The DNA sequence 3' to the termination codon but 5' to the terminal repeats is 186 bases long. Like the 5' region, the 3'-flanking region also contains several possible control signals. The pentanucleotide AATAA occurs downstream from the termination codon at positions 1,412 and 1,424. This sequence may be a potential signal for polyadenylation and is commonly found near the end of the 3'-untranslated region of eukaryotic mRNAs^{14,15}. A sequence that serves as a transcriptional termination signal for type III genes, TTTT¹⁶, occurs 3' to the termination codon at position 1,433. This tetranucleotide also occurs as part of the terminal repeats at the 3' end of the anti-sense strand. As with the CCAAA pentanucleotide in the 5'-terminal repeats, it is unknown whether the TTTT tetranucleotide in the 3' repeats has an actual role in RNA synthesis.

The 3'-flanking region contains a sequence similar to that

* Present address: Department of Genetics and Development, University of Illinois, Urbana, Illinois 61801, USA.

† To whom reprint requests should be addressed.

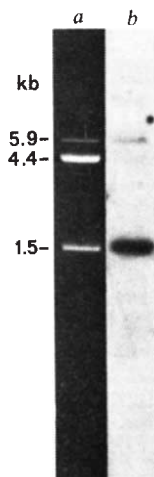


Fig. 4 Hybridization of *O. fallax* RNA to pOfACT(1.6). The plasmid pOfACT(1.6) was digested to near completion with *Pst*I, the fragments were separated by electrophoresis in a 1.2% agarose gel and then blotted on to nitrocellulose. *O. fallax* 7-15S RNA was cleaved by brief alkaline hydrolysis and labelled with 32 P using polynucleotide kinase and 32 P-ATP. Hybridization was carried out for 42 h at 65 °C with 1 M [Na $^{+}$] in conditions described previously⁵. The filter was washed in 2×SSC, 0.1% SDS, 0.1% Na-pyrophosphate at 65 °C. The input RNA was shown to be alkaline hydrolysable, thus ruling out DNA-DNA hybridization. *a*, DNA stained with ethidium bromide showing the *O. fallax* actin sequence at 1.5 kb, pBR322 at 4.4 kb and linear plasmid at 5.9 kb. *b*, Hybridization of *O. fallax* RNA.

The actins are a strongly conserved family of eukaryotic proteins. Actin isolated from the slime mold *Physarum polycephalum*, for example, differs from mammalian cytoplasmic actin in only 17 out of 375 amino acid residues—95.5% homology²⁰. However, the *O. fallax* actin encoded by the 1.6-kb long macronuclear DNA differs considerably in structure from these proteins. It is possible that the *O. fallax* actin has become highly specialized in function. Ciliated protozoans are complex cells having a highly ordered anatomical structure, including such features as ciliary rows, an oral apparatus and a cytoproct (cell anus)²¹. It would not be unusual for these organisms to have proteins modified for use in such specialized structures.

Ciliate actin has not been well characterized. A protein that co-migrates with muscle actin has been isolated from *Tetrahymena thermophila*²² but is absent from *Tetrahymena pyriformis* (N. E. Williams, personal communication). Recently, a fibre-forming protein (FFP-38) has been found in both *Tetrahymena* species²³ that co-precipitates with muscle myosin and is found in contractile regions of the cell. However, FFP-38 is smaller than muscle actin and is biochemically and antigenically distinct. It is possible that FFP-38 may be homologous to the *O. fallax* sequence.

A second *O. fallax* macronuclear DNA molecule has been detected that hybridizes to the yeast actin gene⁵. This molecule is 1.4 kb long and differs substantially in nucleotide sequence from pOfACT (1.6). However, the 1.4-kb molecule hybridizes no more readily to the yeast actin sequence than does the 1.6-kb molecule, and the melting temperatures of the hybrids produced are similar. It is unlikely that the 1.4-kb molecule is any more a true actin gene than the 1.6-kb molecule. Rather, we view the actin genes in *O. fallax* as a multigene family, as in most other eukaryotes²⁴.

The unusual *O. fallax* actin might also reflect a large evolutionary distance between the ciliates and other eukaryotes. Such an interpretation has been supported by other experimental evidence²⁵⁻²⁷ and it has been suggested²⁰ that the ciliates formed a distinct phylogenetic group which may have been the first to separate from the main eukaryotic stem. Our results support this hypothesis.

From the sequence of the *O. fallax* actin gene we conclude that, in hypotrichous ciliates, first, only a small amount of DNA is necessary to make up the noncoding regions of a complete genetic unit, which, in this case is only ~200 bp at each end. Second, the sequenced DNA molecule clearly contains only a single gene. Thus the earlier hypothesis of Prescott and Murti¹ that the macronucleus is a collection of single-gene DNA molecules lacking non-genic spacer sequences seems correct.

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***Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells**

Mary-Dell Chilton*, David A. Tepfer, Annik Petit, Chantal David, Francine Casse-Delbart & Jacques Tempé

Station de Génétique et d'Amélioration des Plantes, INRA, 78000 Versailles, France

Agrobacterium rhizogenes, which induces hairy root disease of dicotyledonous plants¹, is closely related to *Agrobacterium tumefaciens*, the causative agent of crown gall disease¹⁻³. Virulence in both species is conferred by large plasmids⁴⁻⁷. Infected plant tissue synthesizes novel metabolites, opines⁸⁻¹¹, that are not found in normal plant tissues. The pattern of opines synthesized is determined by the type of virulence plasmid in the bacterium, and in general virulence plasmids confer on the host bacterium the ability to catabolize the same opines (refs 8, 12, 13 and A.P. *et al.*, in preparation). Opine synthesis persists when the affected plant tissue is cultivated *in vitro* in the absence of the pathogenic bacterium^{9-11,14,15}, which in the case of crown gall tumours is a consequence of gene transfer from the pathogen to the plant cells. A small specific part of the tumour-inducing (Ti) plasmid of *A. tumefaciens*, termed T-DNA (transferred DNA), is incorporated into host plant nuclear DNA¹⁶⁻²¹ and transcribed into mRNA^{18,22-25}. A specific region of T-DNA confers the ability to synthesize the characteristic opine^{26,27}. Synthesis of opines is thus a natural example of genetic engineering in which the agent is the Ti plasmid of *A. tumefaciens*. The discovery of opines in roots induced by *A. rhizogenes* (ref. 11 and A.P. *et al.*, in preparation) suggested that they too might contain T-DNA derived from the virulence plasmid of the pathogen. We report here DNA hybridization studies that confirm this hypothesis.

A. rhizogenes strain 8196 (from Dr J. Lippincott) contains three plasmids, the intermediate-sized one being the virulence plasmid, isolated in transconjugant C58C1 (pRi 8196) (Fig. 1). Similar data have been reported by White and Nester⁷ for *A.*

* Present address: Department of Biology, Washington University, St Louis, Missouri 63130, USA.

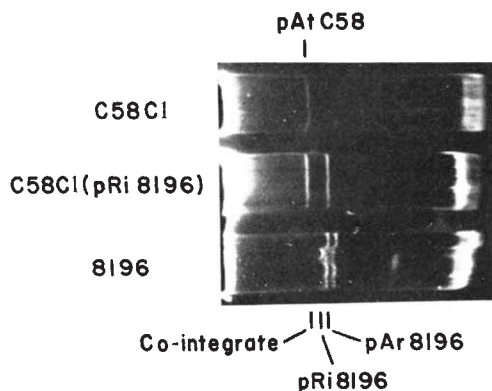


Fig. 1 Eckhardt gel³⁹ analysis of plasmids of *A. rhizogenes* 8196 and C58C1 (pRi 8196). Transconjugant strain C58C1 (pRi 8196) was obtained by conjugation of *A. rhizogenes* 8196 with *A. tumefaciens* C58C1, a strain cured of its Ti plasmid⁴. The donor strain was grown overnight in AT medium⁴⁰ with *N*-(1-mannyl)-glutamic acid (2 g l^{-1})¹¹ as the sole carbon source; the recipient strain was grown overnight in AT medium with mannitol as sole carbon source. Recipient bacteria (10^8) were spread on AT agar with *N*-(1-mannyl)-glutamic acid as sole carbon source, rifampicin ($100\text{ }\mu\text{g ml}^{-1}$) and streptomycin ($500\text{ }\mu\text{g ml}^{-1}$) (drugs to which only the recipient is resistant). Donor bacteria (5×10^7) were applied to the lawn as a drop of liquid culture. Transconjugant colonies were picked after 8 days at 25°C , and cloned on selective medium. Plasmids were visualized by a modification⁴¹ of the Eckhardt gel technique. Strain 8196 contained three plasmids whose relative amounts varied between experiments. The two smaller plasmids (visible here) were sometimes impoverished and a larger plasmid (almost invisible here) predominant. We presume the larger plasmid to be a co-integrate of the smaller ones, analogous to the plasmids of *A. rhizogenes* strain 15824 (ref. 7). The transconjugant strain possesses the larger of the two small plasmids of the donor strain, and is virulent. It also contains the large cryptic plasmid of strain C58C1 (ref. 42).

rhizogenes 15834. As expected, plasmid DNA isolated²⁹ from C58C1 (pRi 8196) exhibits only a subset of the *Bam*HI fragments of plasmid from the parent 8196 strain (see Fig. 2). The giant cryptic plasmid of C58C1 (isolated in extremely poor yield because of its size) is present only as a trace contaminant in this preparation of pRi 8196. We propose the designation Ri for root-inducing virulence plasmids¹¹, analogous to Ti for tumour-inducing plasmids^{30,31}. Contour length measurements by Constantino *et al.*³² have shown pRi 8196 to have a molecular weight (MW) of $137 \pm 2 \times 10^6$.

In our experiments we used carrot disks inoculated with $\sim 10^9$ bacteria in 0.1 ml H_2O . Roots appeared after 2–3 weeks. Terminal pieces 10–15 mm long were excised and planted on Monier medium¹¹ without hormones, solidified with 1–1.5% agar. Explants were incubated in darkness at $25\text{--}27^\circ\text{C}$ and grew vigorously as roots. Those without visible bacterial contamination were transferred every 2–3 weeks and subcultured in Monier liquid medium without hormones (80 ml per Petri dish). Sterility of the cultures was assessed by plating macerates of the cultures on rich medium¹¹. The cultures studied here were derived from single root tips; such cultures could be either cellular clones or chimaeras. Normal root cultures used as controls were established from roots of axenic carrot seedlings.

DNA was isolated from axenic root cultures previously induced by strains 8196 ('8196 root DNA') and C58C1 (pRi

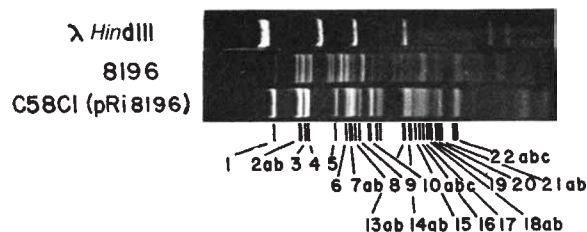


Fig. 2 *Bam*HI cleavage pattern of plasmids from *A. rhizogenes* 8196 and C58C1 (pRi 8196). *Bam*HI digests of plasmid DNA ($2\text{ }\mu\text{g}$ samples) were electrophoresed in a horizontal agarose gel (0.7%)³¹. The line drawing indicates our assignment of fragment numbers, which was based in part on other studies in which digests were electrophoresed for longer.

8196) ('8196TC root DNA'), and from normal carrot roots cultivated similarly. The DNA isolation procedure is described in Fig. 3 legend. Southern blot hybridization analysis was performed on these DNA samples to determine the presence of any fragments homologous to the virulence plasmid. Plant DNA samples digested with *Bam*HI, as well as undigested samples, were electrophoresed in horizontal agarose gels. As controls, two parallel lanes were run with a low concentration of pRi 8196 digested to completion with *Bam*HI; the concentration used was calculated to be the amount of plasmid DNA expected per $5\text{ }\mu\text{g}$ of carrot DNA if the transformed roots contained one or three copies of the virulence plasmid per diploid nucleus. A Southern blot of the resulting agarose gel was hybridized with pRi 8196 DNA labelled *in vitro* by nick translation³³. The autoradiogram of the blot (Fig. 3) shows hybridization bands in the lanes containing digests of transformed root DNA, but not in the lane containing normal carrot DNA. T-DNA was therefore present in the roots transformed by pRi 8196.

Two prominent hybridization bands in the 8196TC root DNA digest co-migrated with bands 6 and 19 of the pRi 8196 digest and with hybridization bands in the 8196 root DNA digest. We interpret these T-DNA bands as 'internal fragments'^{17–21} consisting entirely of pRi 8196 DNA. Six additional faint hybridization bands in 8196TC root DNA have no counterpart in 8196 root DNA, which in turn has four unique bands. We

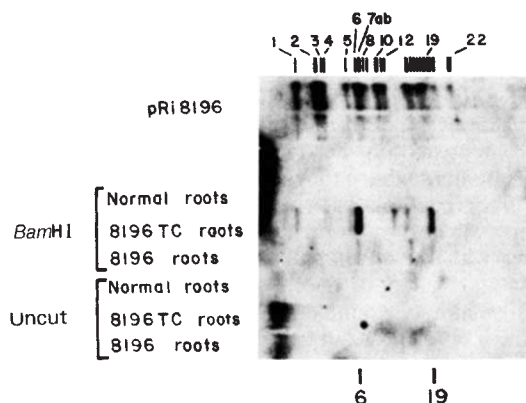


Fig. 3 Southern blot hybridization analysis of transformed root DNA demonstrating T-DNA. DNA was extracted from 40 g fresh weight of roots, collected from liquid culture during vigorous growth. Tissue was frozen in liquid nitrogen and pulverized in a coffee mill pre-cooled with dry ice chips. The powdered tissue was added to an emulsion of 40 ml equilibrated phenol (distilled and shaken with 0.02 M Tris buffer pH 8.0), 10 mM Tris, 50 mM EDTA, 50 mM NaCl, 1 mM CaCl_2 , $400\text{ }\mu\text{g ml}^{-1}$ ethidium bromide, 5 mM β -mercaptoethanol (40 ml) and 25% SDS (2.4 ml). The emulsion was allowed to melt and was stirred at 22°C for 5 min before centrifugation ($17,000\text{g}$, 20 min). The supernatant was removed and again phenol-extracted. DNA was precipitated by addition of sodium acetate (0.3 M final concentration) and 0.75 vol isopropyl alcohol (-20°C for 6 h or overnight). DNA was collected by centrifugation ($17,000\text{g}$, 30 min), re-dissolved in 10 mM Tris, 5 mM EDTA pH 8.0, and was centrifuged to equilibrium in six CsCl-ethidium bromide step gradients in a Spinco SW50.1 rotor ($35,000\text{ r.p.m.}$ at 15°C for 48 h). DNA bands were visualized using long-wavelength UV light and were removed by syringe. After repeated extractions with isopropyl alcohol to remove dye, the DNA solution was diluted with an equal volume of water, adjusted to 0.3 M sodium acetate and precipitated with 0.75 vol isopropyl alcohol. DNA was spooled on a rod, re-dissolved in 10 mM Tris, 1 mM EDTA pH 8, then dialysed briefly against the same buffer before restriction endonuclease cleavage. All glassware used for plant DNA isolation was either new or acid-cleaned and/or autoclaved to eliminate accidental contamination of the sample with native bacterial or plasmid DNA. Plant DNA and pRi 8196 were digested to completion with *Bam*HI (Boehringer-Mannheim). Plant DNA ($5\text{ }\mu\text{g}$ per well), either digested or undigested, and pRi 8196 plasmid DNA digest (1.6 or $0.5 \times 10^{-3}\text{ }\mu\text{g}$) (equivalent to three or one copies, respectively, per diploid carrot DNA equivalent based on a genome of $\text{MW } 1.2 \times 10^{12}$ (ref. 43)) were electrophoresed in horizontal 0.8% agarose gels. DNA was blotted on to cellulose nitrate (Schleicher & Schuell)⁴⁴ and hybridized with pRi 8196 DNA, labelled to a specific activity of $90 \times 10^6\text{ c.p.m. }\mu\text{g}^{-1}$ by nick with [$\alpha\text{-}^{32}\text{P}$]dCTP (Amersham) using a commercial kit (Amersham). Labelled DNA ($1\text{ }\mu\text{g}$) was incubated for 36 h with the Southern blot in $3 \times \text{SSC}$, $5 \times \text{Denhardt's}$ solution at 68°C as described by Thomashow *et al.*⁴⁵. Blots were washed⁴⁵ to remove unhybridized probe and were subjected to autoradiography on Kodak RP X-omat film at -80°C for 8 h with two intensifying screens.

interpret these bands as 'border fragments' analogous to those seen in T-DNA studies of crown gall tumour DNA¹⁷⁻²¹, consisting of T-DNA covalently joined to host plant DNA. The six and four border fragments observed in these autoradiograms suggest that the transformed root cultures contained, respectively, three and two copies of T-DNA. The possibility of additional copies is not excluded. The various T-DNA copies may not be contained in the same cell, as the root cultures used here were not cloned from single cells.

Uncleaved DNA from the transformed roots, but not that from normal carrot roots, hybridized to the labelled probe DNA at the position of the high molecular weight plant DNA in the original gel. T-DNA does not migrate as a sharp band on the gel, as expected for a free replicon of fixed size. A very large free replicon would, however, escape detection because of fragmentation during DNA isolation. Our isolation method would certainly have broken covalently closed circular DNA molecules.

When we used the mixture of plasmids from *A. rhizogenes* strain 8196 (Fig. 2) as probe in a similar hybridization to transformed root DNA digests, we detected the same T-DNA fragments (data not shown), which confirms that all T-DNA is situated on pRi 8196.

Roots containing T-DNA can give rise to plantlets via callus and embryogenesis. Root tissue was transferred to Monier medium containing 2,4-D (0.36 µM) and kinetin (0.72 µM) solidified with agar (0.8%). After 4 weeks, the resulting callus tissue was transferred to liquid hormone-free Monier medium and incubated on an orbital shaker (150 r.p.m.) at 22–25 °C. The callus dissociated into suspension culture, and after 1 month embryos differentiated from these suspensions. Embryos were further incubated in Petri dishes containing hormone-free Monier medium where they developed into plantlets. Adventitious embryogenesis was observed on the newly differentiated plantlets, which resulted in the formation of tufts but did not interfere with the development of the plantlets into vigorous plants of normal appearance.

Of 20 plantlets examined, all contained high levels of the opine characteristic of transformed roots, N²-(1-mannityl)-glutamine (mannopine), which suggests that the transformed roots derived from single root tips may indeed be a cellular clone. Studies of T-DNA in the plants obtained will determine whether each contains the several T-DNA inserts found in the parental root culture.

The results presented here demonstrate that a new family of pathogenic plasmids, the Ri plasmids that induce hairy root disease, share with the Ti plasmids (crown gall agent) the ability to insert T-DNA into the host plant genome. The Ri plasmids studied thus far share little DNA homology with the T-DNA of the Ti plasmids^{34,35}; thus they seem to represent separate pathogenic agents. The similarity of the molecular mechanism of plant cell transformation by Ti and Ri plasmids thus extends the T-DNA concept and suggests that additional examples of foreign gene transfer may be found in plant pathogens.

Because of their natural ability to insert T-DNA into plant cells, the Ti plasmids are being extensively investigated as vectors for use in plant genetic engineering^{36,37}. Regeneration of complete plants from crown gall tissue has proved extremely difficult³⁸. In contrast, the roots containing pRi 8196 T-DNA regenerate reproducibly into plants containing opines, and thus presumably containing T-DNA. The Ri plasmid is thus an attractive alternative to the Ti plasmid as a genetic engineering vector.

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Stratification and terminal differentiation of cultured epidermal cells

Fiona M. Watt*† & Howard Green*†

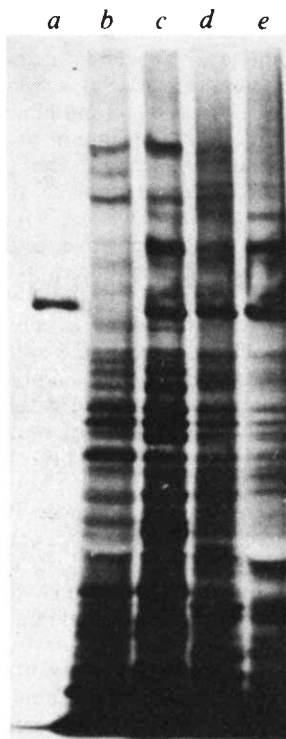
* Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

† Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115, USA

As keratinocytes move from the basal to the outer layers of a stratified squamous epithelium, they enlarge and terminally differentiate. Hence, the stage of terminal differentiation is correlated with both cell size and position. To determine whether position is an important signal in differentiation, we have now grown human keratinocytes in conditions that prevented stratification but not cell enlargement. Involucrin, a marker of terminal differentiation, was synthesized in such a monolayer, but only by large cells. We conclude that the onset of involucrin synthesis, while normally restricted to cells in a non-basal position, does not depend on this position for an essential signal; however, a signal associated with increased cell size is not ruled out. When monolayer cultures were induced to stratify, the large involucrin-containing cells preferentially adopted a suprabasal position, indicating that terminal differentiation is associated with a decrease in substrate adhesiveness. Suprabasal cell position is therefore a consequence rather than a cause of terminal differentiation.

† Present address: Kennedy Institute of Rheumatology, Hammersmith, London W6 7DW, UK.

Fig. 1 Presence of involucrin in keratinocyte monolayers grown in low calcium medium. Second to fourth subcultures of human epidermal cells isolated from newborn foreskin were cultured in the presence of irradiated 3T3 feeder layers⁹ in Dulbecco-Vogt medium supplemented with 10% fetal calf serum, $0.4 \mu\text{g ml}^{-1}$ hydrocortisone, 10^{-10} M cholera toxin¹⁰, $5 \mu\text{g ml}^{-1}$ transferrin, $5 \mu\text{g ml}^{-1}$ insulin and 2×10^{-11} M triiodothyronine³. Epidermal growth factor was added to 10 ng ml^{-1} beginning on the third day after subculture¹¹. The calcium concentration of control medium, determined by flame photometry, was 1.9 mM . When medium was prepared without calcium and supplemented with fetal calf serum that had either been dialysed against Ca-, Mg-free isotonic phosphate buffer (PBS) or treated with Chelex-100 resin¹², the final concentration of Ca^{2+} in the medium was about 0.1 mM (low calcium medium). Cells were labelled before collection with $\sim 15 \mu\text{Ci ml}^{-1}$ ^{35}S -methionine (Amersham, 600–1,300 Ci mmol^{-1}) for 24 h in medium containing 3 mg l^{-1} methionine. They were collected by scraping and sonicated in 10 mM EDTA, 50 mM Tris, $\text{pH } 7.4$. Insoluble material was removed by centrifugation for 2 min at $12,800\text{g}$. *a*, Purified ^{35}S -involucrin marker. *b*, Extract of small cells selected by passage through a Nitex screen. Note the absence of involucrin. Small cells were plated out in control medium (*c*) or low calcium medium (*d*) and collected at confluence. *e*, Cells collected from the medium of a culture in low calcium by gentle aspiration 1 week after confluence. Only cells that were already detached or loosely attached to the monolayer were collected in this way.



Involucrin is a soluble keratinocyte protein that becomes incorporated into the cross-linked envelope during terminal differentiation¹. It is found in all human stratified squamous epithelia and cell cultures derived from them². We have previously shown that the onset of involucrin synthesis is correlated with an increase in cell size³ and with cell migration from the basal layer into the outer layers^{1,2}. In culture, synthesis begins in the layers immediately suprabasal, while in intact tissues, cells migrate several layers beyond the basal layer before starting to make the protein^{1,2}. Hennings *et al.*⁴ have shown that cultivation of keratinocytes in medium containing a low concentration of Ca^{2+} prevents stratification by inhibiting desmosome formation, and this allows the relationship between cell position and involucrin synthesis to be tested.

Pre-confluent cultures of human epidermal keratinocytes derived from newborn foreskin were collected and passed through a screen of Nitex nylon monofilament cloth (type HC-3-11, Tetko). Only cells $< 15 \mu\text{m}$ in diameter passed through the screen; these cells did not contain involucrin, as judged by immunofluorescence and polyacrylamide gel electrophoresis (ref. 3 and Fig. 1*b*).

The small involucrin-free cells were collected in sterile conditions and plated in the presence of irradiated 3T3 cells in medium containing 0.1 mM Ca^{2+} (low calcium medium), grown into a confluent monolayer, then fixed and stained for indirect immunofluorescence, using antiserum raised in rabbits against purified involucrin¹. As Figs 2 and 3 show, even though the cells

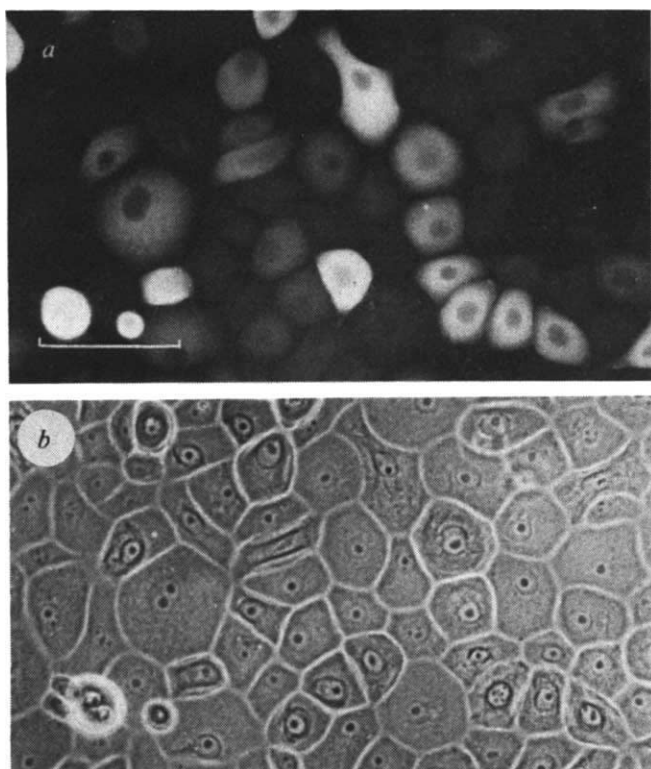


Fig. 2 Identification by immunofluorescence of involucrin-containing cells in keratinocyte monolayers grown in low calcium medium. Keratinocytes grown to confluence in low calcium medium and fixed with 3.7% formaldehyde in PBS (room temperature) for 8 min, in absolute methanol (-20°C) for 4 min and in absolute acetone (-20°C) for 2 min. The cells were incubated first with rabbit antiserum to involucrin (1:20 in PBS), then with fluorescein-conjugated goat anti-rabbit IgG (1:16 in PBS) (Miles). *a*, Immunofluorescence image. *b*, Phase image of the same field; scale bar $100 \mu\text{m}$. Note mixture of involucrin-positive and -negative cells in the monolayer. Positive cells also seem to have better defined nuclear borders.

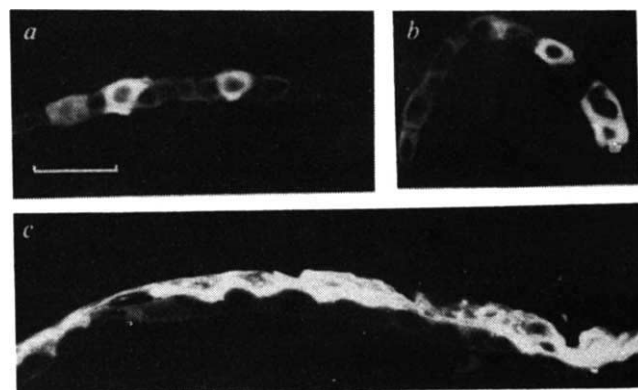


Fig. 3 Selective expulsion of involucrin-containing cells from the basal layer during stratification induced by returning the calcium ion concentration to 1.9 mM . *a*, *b*, Cultures grown to confluence in low calcium medium, then incubated in control medium for 2 h, to allow junction formation without stratification, so that the cells could be detached as an intact sheet with the enzyme dispase¹³. The sheet was fixed in 3.7% formaldehyde at room temperature for 1 h, embedded in paraffin, and $5 \mu\text{m}$ sections were cut at right angles to the surface of the epithelial sheet. The sections were prepared for immunofluorescence as described in Fig. 2 legend. Note that involucrin-positive and -negative cells are interspersed in the monolayer. *c*, Cells grown to confluence in low calcium medium, and transferred to control medium for 24 h before fixation, to induce stratification. The section was prepared as in *a* and *b*. Note that all the involucrin-positive cells have left the basal layer. *a*, *b*, *c* Are all at the same magnification; scale bar, $50 \mu\text{m}$.

were confined to a monolayer, the proportion of involucrin-positive cells was 30–50%, similar to the proportion found in stratified colonies cultured in control medium³. The presence of involucrin was confirmed by polyacrylamide gel electrophoresis of ^{35}S -methionine-labelled cell extracts (Fig. 1, compare *c* and *d*). Thus, terminal differentiation progressed to the stage of involucrin synthesis, even though stratification was prevented by the low Ca^{2+} concentration.

In control medium, the modal cell diameter was $12\text{--}13 \mu\text{m}$ and virtually all cells of $\geq 17 \mu\text{m}$ synthesized involucrin³. Cells grown to confluence in low calcium medium tended to be larger, the modal diameter increasing by $1\text{--}2 \mu\text{m}$ after 1 week in culture. In low calcium medium none of the involucrin-positive

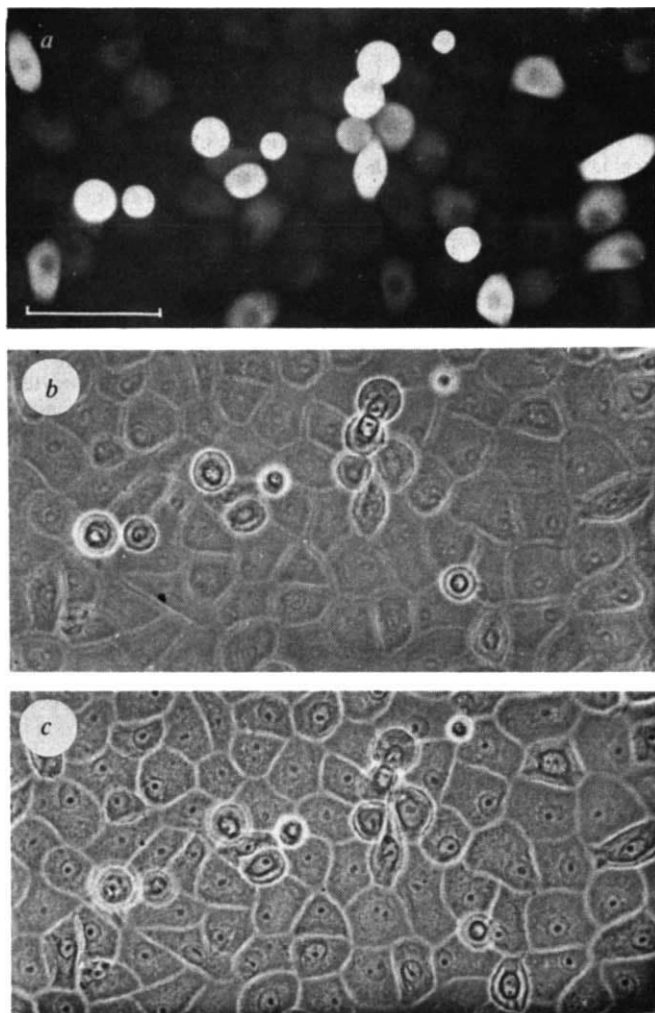


Fig. 4 Selective expulsion of involucrin-containing cells from monolayers maintained in low calcium medium for 1 week after confluence. The cells were prepared as described in Fig 2 legend. *a*, *b*, *c*, Same field: *a* is the immunofluorescence image, *b* is the phase image, focused on rounded cells above the plane of the monolayer, and *c* is the phase image focused on the plane of the monolayer. Note that all the cells lying above the plane of the monolayer contain involucrin. Scale bar, 100 μ m.

cells was $<17 \mu\text{m}$ in diameter, and 66% of cells $>18 \mu\text{m}$ were positive. Although the absolute size at which involucrin synthesis began depended to some extent on the culture conditions, the onset of synthesis in both monolayer and stratified cultures was correlated with an increase in cell size.

We also investigated the effect of returning the calcium concentration to 1.9 mM, thereby inducing stratification, to see whether the involucrin-positive and -negative cells would distribute randomly between the basal and outer cell layers or whether they would sort out. Within 24 h of raising the calcium concentration, stratification occurred (Fig. 3c): all the differentiating, involucrin-positive cells were found in the outer cell layers and the involucrin-negative cells remained in the basal layer. Thus, although the newly stratified cultures were only two to three layers thick, the distribution of involucrin was the same as in cultures grown in control medium². This demonstrates that the cells sufficiently differentiated to synthesize involucrin were selectively expelled from the basal layer.

Monolayers of keratinocytes were also maintained in low calcium medium for 1–2 weeks after reaching confluence. Some cell division continued in the cultures and those cells forced out of the monolayer were rounded and either loosely attached to the surface of the monolayer, or detached into the medium. When such cultures were fixed and stained for immunofluorescence with antiserum to involucrin, it was clear that involucrin

was present in all the rounded cells above the plane of the monolayer as well as in most cells detached and free in the medium (Fig. 4). Polyacrylamide gel electrophoresis confirmed that extracts of ³⁵S-methionine-labelled cells detached from the surface of the monolayer by gentle aspiration contained involucrin (Fig. 1e). This suggests that the terminal differentiation of epidermal cells is accompanied by a decrease in substrate adhesiveness, which could account for the sorting out of involucrin-positive and -negative cells observed when stratification is induced.

These observations show that cells need not achieve a suprabasal position in the stratified epithelium to synthesize involucrin. Rather, the ability of involucrin-containing cells to sort out shows that cell position in the epithelium depends on properties established by the process of terminal differentiation. Selective expulsion of the more differentiated cells from the basal layer has been postulated to explain the kinetics of thymidine labelling in the superficial layers of intact epidermis⁵. Quantitative differences in adhesiveness are known to be sufficient to produce immiscibility between two cell populations⁶. A continuing gradient of surface adhesiveness linked to terminal differentiation and extending outwards from the basal layer would serve to maintain homogeneity of cells along a given plane in the epithelium by preventing the mixing of cells at different stages of differentiation. This is probably the basis for the ability of mixed epidermal cells at different stages of differentiation to reassemble histotypic structure, either in culture⁷ or following their injection into animals⁸.

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Unusual interactions of benzodiazepine receptor antagonists

David J. Nutt & Philip J. Cowen

MRC Unit and University Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, UK

Hilary J. Little

Department of Pharmacology, Oxford University, South Parks Road, Oxford OX1 3QT, UK

Two compounds have recently been described which act as potent benzodiazepine (BDZ) antagonists *in vivo* and which, *in vitro*, show high affinity and selectivity for the BDZ receptor of the mammalian central nervous system (CNS). One, ethyl β -carboline-3-carboxylate (β -CCE), was extracted from human urine and may be related to an endogenous ligand for the BDZ receptor^{1–3}. It reverses the effects of BDZs *in vivo*^{4,5} and *in vitro*⁶, but also has intrinsic activity, as it lowers the seizure threshold to drugs antagonistic to the action of γ -aminobutyric acid (GABA)^{5,7}. The other compound, an imidazodiazepine (Ro 15-1788), which is also a potent and specific antagonist of

BDZ binding *in vivo* and *in vitro*⁸, blocked the sedative, hypnotic and anticonvulsant actions of conventional BDZs, without demonstrating any intrinsic activity^{9,10}. Because of the different profiles of action of these two BDZ 'antagonists', we have here investigated their interactions in two well established systems for assessing BDZ activity: seizure thresholds *in vivo*¹¹ and the action of GABA on cervical sympathetic ganglia *in vitro*¹². We find that Ro 15-1788 not only opposes the actions of BDZs but also is an effective antagonist of β -CCE in both systems. At high doses it has BDZ-like activity, suggesting that it may be a partial agonist at the BDZ receptor site.

Seizure thresholds in rats were determined by measuring the dose of drug, when infused at a constant rate through a tail vein, needed to elicit a seizure (see Table 1 legend), a method known

Table 1 Effect of different doses of Ro 15-1788 on the seizure thresholds to PTZ, bicuculline and strychnine

Dose of Ro 15-1788 (mg per kg)	PTZ	Seizure thresholds (mg per kg) Bicuculline	Strychnine
Vehicle alone	30 $\pm$ 4 (5)	0.24 $\pm$ 0.02 (4)	2.2 $\pm$ 0.4 (5)
1	—	0.25 $\pm$ 0.02 (4)	—
10	29 $\pm$ 2 (6)	0.26 $\pm$ 0.03 (4)	—
50	36 $\pm$ 5 (6)*	0.29 $\pm$ 0.06 (4)*	2.1 $\pm$ 0.3 (6)
100	40 $\pm$ 5 (5)†	—	—

Seizure thresholds are expressed as mean $\pm$ s.d. with number of animals in parentheses. Rats were given either vehicle or Ro 15-1788 in a volume of 2 mg per kg i.p. and 15 min later convulsant was infused in a tail vein via a 25-gauge cannula, at a rate of 2.22 ml min⁻¹, and the time taken to the first seizure activity was recorded. Using this time and the concentration of infused drug and weight of the animal, the seizure threshold was computed. The concentrations of convulsant solutions used were PTZ 10 mg ml⁻¹, bicuculline 0.05 mg ml⁻¹ and strychnine 1 mg ml⁻¹. PTZ and strychnine were dissolved in normal saline; bicuculline was dissolved in saline acidified to pH 3 with 0.1 M HCl. Significance was determined by Student's *t*-test: * *P* < 0.05; † *P* < 0.01, as compared with vehicle controls.

to provide a reliable guide to the activity of certain anticonvulsant drugs¹³. Ro 15-1788 was prepared as a suspension in distilled water (DW) with 1 drop of Tween 40 per 10 ml and was injected intraperitoneally (i.p.) 15 min before infusion. All animals received a volume of 2 ml per kg, controls being given the Tween/DW vehicle.

When tested alone on seizure thresholds to the GABA antagonist drugs pentylenetetrazol (PTZ) and bicuculline, Ro 15-1788 showed anticonvulsant activity at a dose of ≥ 50 mg per kg, with a potency about 1/50th that of diazepam (Table 1). However, even at 50 mg per kg, Ro 15-1788 gave no protection against the seizures produced by the glycine antagonist, strychnine, suggesting that Ro 15-1788's anticonvulsant effect has a similar pharmacological specificity to that of the BDZs¹⁴.

To determine the effect of Ro 15-1788 on the anticonvulsant effect of diazepam, rats were injected with diazepam, 5 mg per kg, and 15 min later with Ro 15-1788, 10 mg per kg (this dose was devoid of agonist effects, see Table 1). When PTZ was infused a further 15 min later the pronounced elevation of seizure threshold produced by diazepam was substantially reduced in the Ro 15-1788-treated group (Table 2).

The selectivity of this effect was determined by giving the same dose of Ro 15-1788 15 min after treatment with anticonvulsants thought to act at sites other than the BDZ receptor. As Table 2 shows, the anticonvulsant activity of phenobarbitone (40 mg per kg, i.p.), sodium valproate (400 mg per kg, i.p.) and progabide (200 mg per kg, i.p.), which raise seizure thresholds to PTZ by mechanisms which may involve GABAergic systems¹⁵⁻¹⁷, was unaffected by Ro 15-1788.

The action of Ro 15-1788 on the previously reported proconvulsant action of β -CCE was evaluated by administering Ro 15-1788, 10 mg per kg, or Tween followed 10 min later by intravenous (i.v.) β -CCE, 1 mg per kg (1 mg ml⁻¹ in propylene glycol/ethanol 2:1) and 5 min after this by PTZ infusion. The fall in seizure threshold produced by β -CCE was abolished by the Ro 15-1788 pretreatment (Table 2).

In the seizure threshold model, therefore, Ro 15-1788 at a dose of 10 mg per kg i.p. antagonized both the anticonvulsant effects of 5 mg per kg diazepam and the proconvulsant effects of 1 mg per kg β -CCE, without having any detectable effect of its own. This antagonistic effect seems to be selective for drugs acting at the BDZ receptor, as Ro 15-1788 did not reduce the anticonvulsant effects of phenobarbitone, sodium valproate or progabide.

GABA perfusion of the isolated rat cervical sympathetic ganglion induces depolarization which may be blocked by the GABA receptor antagonist, bicuculline. In this preparation the conductance change produced by GABA (an increase in chloride conductance) has been shown to correspond with those produced by GABA in the CNS¹⁸. Moreover, the binding characteristics of the ganglion and CNS GABA receptors are similar¹⁹. Figure 1 shows that, in agreement with previous studies¹² with BDZs, the water-soluble BDZ, chlordiazepoxide, increases the amplitude of the responses to GABA in the presence of bicuculline. Treatment of the ganglion with Ro 15-1788 (835 nm) abolished the effect of chlordiazepoxide (Fig. 1),

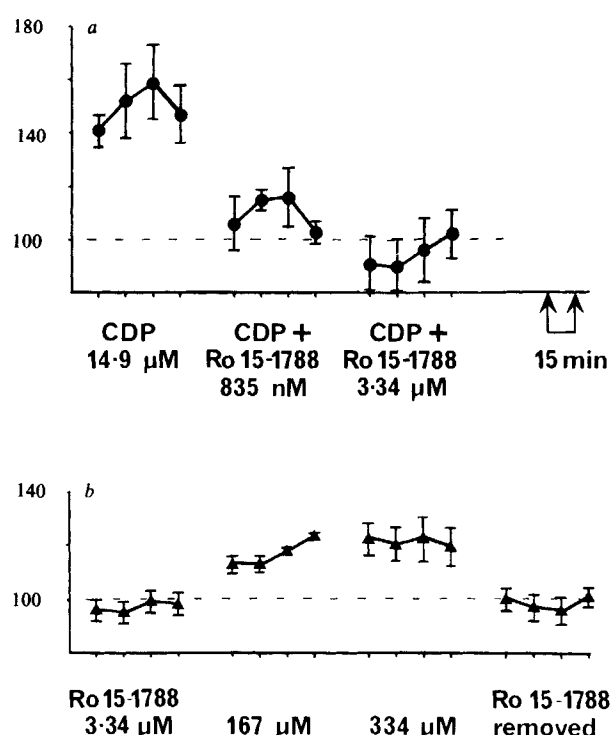


Fig. 1 The method used was that of Brown and Marsh²² and involves superfusion of the isolated ganglion (1 ml min⁻¹) with extracellular recording of the postsynaptic potential changes. Application of GABA to the perfusion fluid for 2 min every 15 min was found to give steady control responses without desensitization. Four steady control responses to GABA (38.8 μ M) were obtained in each experiment before the above treatments were applied. This concentration produces near-maximal responses to GABA in this preparation and the concentration of bicuculline used throughout (27.2 μ M) reduced this to approximately half the maximum responses. These concentrations were similar to those used originally by Bowery and Dray¹² and were chosen to demonstrate the optimum changes in response amplitude. The results give the amplitude of the depolarizations produced by application of GABA, expressed as percentages of the mean of the four control values (*n* = 5 throughout). The compounds were added to the perfusion medium 15 min before GABA and were present throughout the treatment periods. Standard errors are included to give an indication of the variation. Analysis of variance performed on the original data showed that Ro 15-1788 (concentrations 835 nM and 3.34 μ M) significantly antagonized the effects of chlordiazepoxide (CDP, *P* < 0.01). When applied alone, Ro 15-1788 caused significant increases in amplitude of responses at 167 μ M and 334 μ M (*P* < 0.01) but not at 3.34 μ M. There was no further increase in response amplitude when the Ro 15-1788 concentration was raised to 835 μ M. The mean amplitude of the control responses was 211 $\pm$ 19 μ V. The drugs were made up in the following solutions: GABA and Ro 15-1788 in standard Krebs, chlordiazepoxide in distilled water, β -CCE in 0.03 M HCl, bicuculline in 0.1 M HCl titrated to pH 3.

Table 2 Effect of Ro 15-1788 on the changes in seizure threshold produced by diazepam, other anticonvulsants and β -CCE

Drug treatment	Seizure threshold to PTZ (mg per kg)		
	Vehicle (Tween)	Ro 15-1788 (10 mg per kg)	P value
Saline	34 $\pm$ 2 (5)	35 $\pm$ 3 (6)	N/S
β -CCE (1 mg per kg, i.v.)	26 $\pm$ 3 (5)	35 $\pm$ 3 (6)	<0.001
Diazepam (5 mg per kg, i.p.)	62 $\pm$ 14 (7)	39 $\pm$ 4 (5)	<0.001
Phenobarbitone (40 mg per kg, i.p.)	52 $\pm$ 5 (6)	56 $\pm$ 9 (6)	N/S
Valproate (400 mg per kg, i.p.)	57 $\pm$ 14 (5)	53 $\pm$ 10 (6)	N/S
Progabide (200 mg per kg, i.p.)	50 $\pm$ 6 (7)	55 $\pm$ 11 (7)	N/S

Diazepam, phenobarbitone, sodium valproate and progabide were given in the doses shown. Concentrations of solutions were diazepam 5 mg ml⁻¹ in commercial vehicle (Roche), phenobarbitone 10 mg ml⁻¹ and sodium valproate 100 mg ml⁻¹ in normal saline, and progabide 100 mg ml⁻¹ in Tween. Saline was given in a volume of 4 ml per kg. 15 min later Ro 15-1788, 10 mg per kg, was injected i.p. and a further 15 min later PTZ was infused (see Table 1 legend). With β -CCE, Ro 15-1788, 10 mg per kg in Tween, was given 10 min before an i.v. injection of 1 mg per kg β -CCE (1 mg ml⁻¹ solution), with PTZ infused 5 min later. Seizure thresholds were expressed as mean $\pm$ s.d. with the number of animals given in parentheses. The P value refers to significance of differences between Ro 15-1788 and Tween vehicle-treated animals. There were no differences in seizure threshold between animals pretreated with saline, Tween, β -CCE vehicle or diazepam vehicle. Statistical analysis performed by Student's *t*-test. N/S, not significant.

while higher concentrations of Ro 15-1788 (167–835 μ M) showed intrinsic activity in that they increased the responses to GABA (Fig. 1). The ratio of agonist and antagonist doses of Ro 15-1788 in this system was similar to that displayed in the seizure threshold model.

Barbiturates also increase the responses to GABA in the ganglion preparation¹², but as in the seizure threshold model, the effect of phenobarbitone (40% increase in the amplitude of GABA responses at 787 μ M) was not antagonized by Ro 15-1788 at either 3.34 or 33.4 μ M. These concentrations of Ro 15-1788 alone had no effect on the response to GABA.

When applied to the GABA–bicuculline-pretreated ganglion, 1 μ M β -CCE showed opposite actions to BDZs in that it significantly ($P < 0.01$) decreased the depolarization produced by GABA, 38.8 μ M (results, expressed as in Fig. 1, were 85 $\pm$ 3% at 15 min, 78 $\pm$ 5% at 30 min, 85 $\pm$ 6% at 45 min and 83 $\pm$ 7% at 60 min). This effect was antagonized by Ro 15-1788 at the same concentration which blocked the effect of chlor-diazepoxide (3.34 μ M).

It thus seems that in two experimental systems in which specific BDZ activity may be assessed, β -CCE has the opposite effect to BDZs. Ro 15-1788 seems to be a selective and potent antagonist of both compounds in both systems. Investigations have failed to demonstrate specific binding of either β -CCE or Ro 15-1788 (ref. 8) to CNS sites other than the BDZ receptor. In view of the selective and high-affinity BDZ receptor binding of β -CCE and Ro 15-1788, and the parallels between their binding and pharmacological activity, it is likely that the pharmacological effects observed are due to interaction at the BDZ receptor site.

We suggest three possible explanations for these findings. First, the partial agonist effect of Ro 15-1788 may be sufficient to account for its reversal of the actions of β -CCE. This seems unlikely because the concentrations of Ro 15-1788 that antagonized β -CCE had no agonist action when tested alone. However, it is possible that in the presence of β -CCE the BDZ receptor is altered so that Ro 15-1788 has a greater agonist effect.

The second explanation is that the functional interaction of ligands with the BDZ receptor may be unusual in that specific high-affinity ligands may produce opposite pharmacological effects and yet have a common antagonist. The possible existence of this type of receptor interaction has been predicted from receptor theory (see, for example, ref. 20). The allosteric models of receptor behaviour suggest that receptors exist in equilibrium between two states (which may be termed 'active' and 'inactive') and that this equilibrium is altered by ligand binding. If we apply this theory to our findings, BDZs would be

considered to be agonists, binding to the active state, whereas Ro 15-1788 would be a conventional antagonist, binding to both forms without altering the equilibrium. If β -CCE bound preferentially to the receptors in the inactive state it would shift the equilibrium in the opposite direction. This form of antagonism has been predicted but not so far demonstrated.

A third explanation derives from results of ligand binding studies suggesting the existence of subclasses of BDZ receptors. These have identical affinities for the BDZs, including Ro 15-1788 (ref. 8), but differ in their affinities for the β -carbolines^{2,21}. The pharmacological actions of the BDZs and β -CCE may thus be exerted through different subpopulations of BDZ receptors, and it is conceivable that the unusual interactions we have described represent a functional consequence of this differential binding.

We thank Dr B. Jones, Glaxo, and Dr Ian Martin, MRC Neurochemical Pharmacology Unit, Cambridge, for β -CCE, Dr W. Haefely, Roche, for Ro 15-1788, Synthelabo (Paris) for progabide, Professors Sir William Paton and D. G. Grahame-Smith and Dr A. R. Green for helpful advice and criticism and Mr C. Howard for technical assistance.

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Errata

In the *Nature Directory of Biologicals* 1982, on page vi James Manley of Columbia University is incorrectly listed as Michael Manley under the heading 'Scientific Advisory Committees'.

In the letter 'Tree remains in a North York Moors peat profile' by I. G. Simmons & J. B. Innes, *Nature* **294**, 76–77 (1981), Figs 1 and 2 are transposed. Thus Fig. 1 is the pollen diagram shown on page 78.

In the article 'Magma chamber profiles from the Bay of Islands ophiolite complex' by J. F. Casey & J. A. Karson, *Nature* **292**, 295–301 (1981), NAM in Fig. 1 legend stands for North Arm Mountain. The received date for the article should read 29 September 1980.

Corrigendum

In the letter 'Mouse IgG3 antibodies are highly protective against infection with *Streptococcus pneumoniae*' by D. E. Briles *et al.*, *Nature* **294**, 88–90 (1981), the dose of *S. pneumoniae* is given as 100 CFU in Table 2 and 150 in the text. This should read 100 CFU throughout.

BOOK REVIEWS

Tomorrow, the world

Brenda Maddox

ANY writer who sits down to try to explain the public policy issues embedded in complicated technology has two contradictory censors inside his head. "Don't get it wrong", demands one. "Don't make it boring", shouts the other. Because the dilemma is so common, a common solution has evolved: to be lofty. Cast the questions in terms of the survival of the race and the future of the planet; the technical experts will thus forgive you for skating over tedious details, while the lay reader will work extra hard and re-read tough pages rather than giving up.

To be sure, Mr McPhail, who teaches mass communication and journalism at Carleton University in Ottawa, has set his chisel to the hardest stone: Unesco and the issue of the New World Information Order. For at least a decade, the countries of the developing world have been demanding a New World Information Order. This fuzzy concept expresses a legitimate grievance — that most of their communication with the developed world flows in one direction only: in. Little news about them of their own choice flows out toward the wider world, and even less entertainment. They fear eventual cultural swamping and, at the present, resent what is seen as constant misinterpretation by Western news agencies.

To right this balance, these countries are using their numerical strength in the United Nations where each country has one vote. Their favourite battleground is Unesco. Unesco's secretariat has so taken up the Third World's campaign for a re-ordering of information (of news-gathering in particular), that it has come up with proposals which to Britain, the United States and other countries committed to a free (well, fairly free) press amount to nothing less than a Unesco endorsement of government control of the media. In 1980 at Unesco's general conference in Belgrade the West reluctantly, with many doubts and qualifications, allowed the barest acknowledgement that something like a New World Information Order may be needed.

All this sets a very demanding exercise for a writer, especially as Unesco obscures its own activities behind dense screens of jargon. Earnestness so permeates Mr McPhail's efforts that it is sad to have to declare that his book does not meet the challenge. For example, what did the West surrender in the way of press freedom at Unesco's general conference in Belgrade in 1980? The Western press tried analysis (duly quoted in Mr McPhail's text) and was

Electronic Colonialism: The Future of International Broadcasting and Communication. By Thomas L. McPhail. Pp. 260. Hbk ISBN 0-8039-1602-7; pbk ISBN 0-8039-1603-5. (Sage: 1981.) Hbk \$22.50, £15.50; pbk \$9.95, £6.50. *Global Talk: The Marriage of the Computer, World Communications and Man.* By Joseph N. Pelton. Pp.336. Hbk ISBN 0-7108-0371-0; pbk ISBN 0-7108-0347-8. (Harvester: 1981.) Hbk £17.95; pbk £7.95.

hampered by the differing views, even between Britain and the United States, as to what happened. For its part, the developing world is not sure what it has achieved in securing within Unesco the creation of the International Programme for the Development of Communications: a talking shop, a funnel for practical aid or its own power base inside the secretariat? And neither is Mr McPhail. All he can conclude is that the New World Information Order is not going to go away.

For a book on the future of the media, Mr McPhail gets off to a disastrous start. In his very first two words, he spells a man's name wrong — and not just any man, but the legendary Lord Copper, the archetypal Fleet Street press baron of Evelyn Waugh's *Scoop*. Mr McPhail calls him Lord Cooper, somewhat spoiling Waugh's point. He then gets others wrong (President Kekkonen of Finland) but these could be the computer typesetting.

Another device which Mr McPhail's book would have been better without is the use of ponderous lists, with scarcely a verb at all:

The questions of transnational data flows, computers, data processing, privacy, and computerization effects on the work place and labor are central to policy concerns of several industrialized nations.

The third stylistic flaw which makes the book unreadable is the excessive use of long quotations, with little guide as to how the extracted material furthers the author's argument.

At the start of his book, Joseph Pelton looks like falling into the same traps. *Global Talk*, he declares, "is only my third book on science policy and applications". But it was his hardest — because he says he has tried to be funny, and sophisticated too, without being as tedious as the *New York Times*. What a relief, therefore, to wade in and find that Mr Pelton, an executive of Intelsat, the International Telecommunications Satellite Organization, has assembled much hard-to-get and fascinating information —

studies of telecommunications plans and expenditure in developing countries; similar statistical profiles of "the information societies" and on the technical and economic outlook for satellites.

Mr Pelton, too, dips into the slough of the UN conference — specifically the WARC's, the world radio conferences of the International Telecommunications Union. He knows, unlike Mr McPhail, that the "A" in WARC stands for "administrative" and not "administration" and he is, moreover, unafraid to use his own words, instead of quoted material, to draw conclusions. In the case of the 1979 WARC, his judgement is that, for their own purposes, more and more countries are willing to tax the ITU's cumbersome regulatory mechanisms — such as the mammoth WARC conferences — to the maximum, yet he sees this as no cause for despair. In spite of their complexity, the issues of international management of technology are no harder than those which international institutions have dealt with successfully in the earlier part of the century. Not a bold conclusion, perhaps, but a clear one.

In sum, Mr Pelton's grasp of his subject triumphs over his whimsy (although, in some cases, just barely: an excellent chapter on the worsening problem of incompatible international technical standards contains an awful poem, "Electronic Gumbo Stew"), and has allowed him to produce an ambitious and original work of reference. □

Brenda Maddox is Home Affairs Editor of The Economist and Chairman of the Association of British Science Writers. She is author of Beyond Babel: New Directions in Communications (1972).

Tasteless behaviour

Mark Ridley

The Foundations of Ethology. By Konrad Z. Lorenz. Pp.380. ISBN 0-387-81623-2. (Springer-Verlag: 1981.) \$21.95.

The Foundations of Ethology, a translation of a book that appeared in German a few years ago, is a monograph of Lorenzian ethology. The text reads well, unlike most translations, although "selfish", which becomes *egoistische* in German, returns in "The egoistical gene" has become the popular phrase. . . . All in all there is not much about natural selection

in the book, for Lorenz founds ethology in the philosophy of *Gestalt* psychology, in physiology and in embryology. These three fill the main sections of the book: one on philosophy and methods, and two marked by Lorenz's controversial distinction between innate and acquired information. He repeats once again his argument for a fundamental distinction between innate and acquired information, and, in the present environment of debates about "genetic determinism", it is undoubtedly worth repeating. The fact that the behaviour of animals is adapted to their environments means that they must have "information" about their environment; it is this information (not behaviour) which Lorenz dichotomizes into genetic and acquired.

Lorenz could have written a book with the conceptual content of *Foundations of Ethology* 30 years ago. If it had been published then it would have become a classic alongside Tinbergen's *Study of Instinct* (Clarendon, 1951). Coming out now it appears awkwardly antediluvian. In his foreword, T.H. Bullock says that the book is intended as a contribution to the history of ethology. But to write a period textbook is not to write the history of a period. *Foundations of Ethology*, however, is not even simply a period textbook, for Lorenz is far too concerned with defending his concepts against later criticism. The fault with the book can be

clarified by an analogy with historiography. By tracing back the pedigree of ideas that are interesting now, it is easy to distort history, because isolated ancestral ideas are rarely representative of contemporary thinking. Lorenz, by tracing forward and defending his ideas, produces an extraordinary futuristic distortion. For example, in his discussion of motivation he defends himself against Robert Hinde's criticisms and even presents an updated hydraulic analogy. But modern work on motivation is established on quite separate foundations, manifesting itself to most of us only as a fascinating and imposing castle, with sheer walls of solid algebra, surrounded by minefields of exegesis.

Lorenz's later books have tended to be increasingly concerned with human beings, but this one is almost entirely about animal

behaviour. There is a typically nonconformist appendix on the uniqueness of mankind and a stricture or two against the deadly sins of civilized man. Our "innate releasing mechanisms", we are told on p.166, give us an unhealthy liking for chocolate and "even the most complete insight into the workings of our IRMs does not make it easy to avoid suicide by overeating". As a gastronomic experience, *Foundations of Ethology* is not so much suicidal as dry and unappetizing; it rather resembles three full courses of stale bread. Well before I had reached p.166, I had found that it went down best when taken with *religieuses* and *éclair au chocolat*. □

Mark Ridley is a Junior Research Fellow of Oriel College, and a member of the Animal Behaviour Research Group, University of Oxford.

Galactic sociology

John D. Barrow

Galactic Astronomy: Structure and Kinematics, 2nd Edn. By Dimitri Mihalas and James Binney. Pp.597. ISBN 0-7167-1280-6. (W.H. Freeman: 1981.) \$29.95, £20.70.

THE story is told of a lecturer beginning his address on the structure of stars with the words "Stars appear to be very simple objects", whereupon someone in the audience retorted "You'd look pretty simple too at a distance of a hundred light years!". Because they are a good deal farther away than stars, astronomers used to think that galaxies were even simpler. Until very recently elliptical galaxies were thought to be uncomplicated and understood — just isothermal "gases" of stars whose various degrees of ellipticity were merely reflections of their differing rotation rates. During the past six years this naive picture has been obliterated and replaced by that complexity, masquerading as simplicity, that is the hallmark of all natural phenomena. Ellipticals have been revealed as complicated systems with three-dimensional dynamics that are neither isothermal nor rotating to any significant degree. Their flattening is a consequence of much more subtle dynamical properties.

Surprisingly, there has never been an authoritative advanced textbook on the structure of galaxies. There are indeed several good introductory texts and collections of review articles, either scattered around the literature or gathered into massive review volumes that send a shiver down your cheque book, but no single, detailed and up-to-date guide to all the relevant observational data and theoretical ideas — until now.

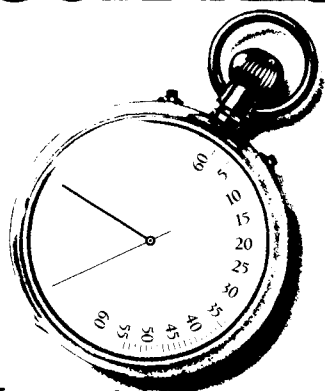
Mihalas and Binney have completed the first part of a two-volume treatise on the subject, this book being confined to obser-

vations and kinematics. The second volume, to be written by Binney and Tremaine, will be devoted to dynamics. By any standards this first volume is outstanding. Although advertised as a second edition of Mihalas and Routly's book of the same title, do not be misled, this is an entirely new book of more than double the length and considerably more depth than its prototype. After a brief history and introduction to astronomical concepts and vocabulary, it begins with a detailed overview of stellar evolution and the interstellar medium before describing the stellar content and kinematics of external galaxies and the Milky Way. The final chapters provide studies of the hydrogenic content of galaxies and their rotational properties. References to the original literature are always provided, although through a rather cumbersome system, and good tables and diagrams illuminate the text throughout.

This book is essential for any graduate course on galaxies. It is too advanced for undergraduate courses although lecturers and more able students will want to refer to it. Most graduate courses on galaxies divide their time equally between the material covered in this volume and the dynamical theory that will appear in Vol. 2, and together the two books should provide everything such students require. Unfortunately, it is hard to believe that many of them will be willing or able to pay the considerable price of both volumes — one hopes that a paperback edition will soon be available, as all astronomers should own and read this book. Perhaps also the authors and publishers might consider a third volume which would be an abridged combination of the two earlier volumes, with a length and level comparable to the original edition written by Mihalas and Routly. □

John Barrow is a Lecturer in Astronomy at the University of Sussex.

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P.W. Hawkes

Optical Information Processing: Fundamentals. Edited by S.H. Lee. Pp.308. ISBN 3-540-10522-0. (Springer-Verlag: 1981.) DM 99, \$42.20.

How is optical information processing getting on? For nearly two decades now, the advantages of light for manipulating data have been attracting more and more attention, although, as Casasent says in this book, "optical processors are somewhat like discrete transistors before the dawn of LSI; they are truly in their infancy". These advantages may be summarized as parallel processing, high throughput and the ability to perform such operations as cross-correlation virtually instantaneously. Such features clearly make optical processors serious potential competitors of digital systems, though, to quote Casasent again, "the shortcomings of one system are the advantages of the other. The two technologies (optical and digital) are actually complementary rather than similar".

The present text, which should be regarded as a companion to an earlier volume in the same Springer series edited by Casasent (*Optical Data Processing: Applications*, 1978), gives an impressively complete account of the degree of sophistication that such optical systems have reached. About a third of the book has been written by the editor, S.H. Lee, who first sets out the basic principles, covering essentially the Fourier transforming properties of lenses and their mathematical implications. His chapter on coherent optical processing then gives an account of filter design and synthesis, including optical feedback, and classifies the various thick and thin filters; a number of more elaborate processor designs are described and clearly illustrated, and the chapter ends with a few words on coherent noise suppression.

The third chapter, by W.T. Rhodes and A.A. Sawchuk, deals with incoherent optical processing, where the advantages of easy input/output, tolerance towards dust and other common imperfections of laboratory equipment and the absence of phase problems is offset by the restriction to real, non-negative signals. This is followed by an extremely important contribution by G.R. Knight on interface devices and memory materials. If the progress described here is maintained, optical processors cannot fail to gain in importance even though an immense amount remains to be done. Some of the problems of purely optical processors can be circumvented by using hybrid systems, as D. Casasent explains in the next chapter. Here, the most powerful features of optical and digital systems are united so that the fast two-dimensional signal handling of the optical unit complements the flexibility of the digital computer. More space might

have been given to the achievements of Lohmann's school in Erlangen, which has made many interesting contributions in this field.

The penultimate chapter, by J.W. Goodman, deals with a difficult task on which comparatively little work has been done, linear space-variant optical data processing. Crudely speaking, here we are concerned with integrals that are not in the form of convolutions and hence do not separate conveniently under Fourier transformation. Goodman's clear statement of the problems involved is particularly welcome. The book concludes with a further contribution by the editor, on nonlinear optical processing: "logarithm, exponentiation, intensity level slicing, thresholding, analog-to-digital conversion, logics and bistability", the last four of which are "important toward developing a futuristic digital optical processor".

Applications of these various techniques are only mentioned in passing, as we should expect in a book on "fundamentals", but many are explored in detail in Casasent's earlier volume which contains chapters on optical transforms and systems, enhancement and restoration, synthetic aperture radar (one of the first triumphs of optical processing), photogrammetry, non-destructive testing and metrology, biomedical applications and signal processing. Taken together, the two books give an excellent account of the present state of this field, still young but rapidly maturing. A minor criticism is the under-representation of European work in both works — perhaps this can be rectified in a future volume in the series. Even so, they give the best available account of a mass of new material, written by experts on the various topics. □

P.W. Hawkes is *Maitre de Recherches* at the *Laboratoire d'Optique Electronique du CNRS, Toulouse*.

Myriapod carnivores

John A. Wallwork

The Biology of Centipedes. By J.G.E. Lewis. Pp.476. ISBN 0-521-23413-1. (Cambridge University Press: 1981.) £33, \$69.95.

FEW students of the soil fauna can fail to be fascinated by centipedes, although these elusive predators are often overlooked because of their secretive habits. Accounts of their biology have hitherto been widely scattered in the literature, usually in a fragmentary fashion. There has long been a need to gather this information together,

stemming from the recognition that these carnivores may regulate the prey populations on which they feed. The prey (mainly Collembola) are principally primary consumers of dead organic material, and predation by centipedes on these "key industry" detritivores may operate to control, indirectly, rates of organic decomposition.

In this book, Dr Lewis does not concern himself with this latter theme to any great extent; indeed, by his own admission, his ecological survey is less than comprehensive. In this, however, he is a victim of circumstances since the integration of centipede ecology with that of the entire soil-litter system is only just beginning. What he has provided is a comprehensive account of centipede biology which soil ecologists will find most useful, and which forms a worthy complement to Eason's earlier taxonomic treatise, *Centipedes of the British Isles* (Warne, 1964). Eason, however, dealt only with British centipedes, and Dr Lewis's experience with temperate and tropical myriapods is reflected in a widely based survey of centipede anatomy, behaviour, physiology, reproduction, life histories, taxonomy and ecology. He devotes as much attention to the mainly tropical Scolopendromorpha as he does to the temperate Lithobiomorpha and the cosmopolitan Geophilomorpha.

As the dust cover of the monograph reminds us, this is primarily a source-book and the way in which Dr Lewis organizes his information — into sections dealing with taxonomic groups — emphasizes the point. Although this device allows the reader easy access to the subject matter, since it is repeated chapter after chapter, the result makes for less than compelling reading; this is a book to inform rather than to be contemplated at leisure. The style is direct, as befits a rather clinical presentation of a catalogue of facts, and the illustrations are numerous but variable in quality, which may reflect the fact that they were taken directly from original sources rather than re-drawn. The reproduction of Manton's (1965) figures are, for example, excellent as might be expected. At the other extreme, the figure of *Lithobius forficatus* eating a fly (Fig. 126) is less than impressive and perhaps unnecessary. Some figures are inadequately labelled (for example, Figs 162a, 203b, 204 and 206). The bibliography is extensive and up to date, and although there is no author index a detailed subject index is provided.

My overall verdict is that the author has succeeded admirably in what he has set out to do. Despite its defects, many of which are unavoidable, the result is a valuable reference book which will be sought by undergraduates and research workers alike. □

John A. Wallwork is *Reader in Zoology* at *Westfield College, University of London*.

Everything you wanted to know about protein phosphorylation

P.J. England

Protein Phosphorylation. Cold Spring Harbor Conferences on Cell Proliferation, Vol. 8. Edited by Ora M. Rosen and Edwin G. Krebs. Two-volume set, pp.1,421, ISBN 0-87969-140-9. (Cold Spring Harbor Laboratory: 1981.) \$140 US, \$168 elsewhere.

THE discovery that enzymes could be reversibly phosphorylated, with concomitant changes in activity, was made 25 years ago. For some time after that it appeared that only the enzymes of glycogen metabolism were so regulated, and that protein phosphorylation was a rather special and restricted form of control. However, during the past decade it has become apparent that almost every aspect of eukaryotic cell function is regulated in some way by phosphorylation, and the phenomenon is now known to have implications in many areas of molecular biology. This rapid expansion has made it difficult even for specialists to keep in touch with all aspects of the subject. The publication of these impressive books is thus most welcome; details of almost all of the current research on protein phosphorylation are brought together, so that no one working in this or related areas now has any justification for ignorance.

The books are the result of a five-day conference held in the summer of 1980. It was attended by about 200 people from all the major laboratories working on protein phosphorylation in North America, and a small number from elsewhere. Approximately 100 talks were given, each one of which is represented by a chapter. These individual contributions are grouped into ten sections, the titles of which give a clear idea of the wide range of topics covered in the two volumes — cyclic AMP-dependent protein kinases: structural studies; cyclic nucleotide-dependent and independent protein kinases and protein phosphorylation; glycogen synthetase/phosphoprotein phosphatases; regulation of lipid and carbohydrate metabolism; insulin and growth factor-promoted protein phosphorylation; contractile proteins; protein synthesis; nuclear and cytoskeletal protein phosphorylation; viruses and cell transformation; neural and membrane protein phosphorylation.

Each of the sections contains a wealth of recent results and interpretations from several laboratories. For example, one of the newest areas of interest is that of tyrosine phosphorylation coupled to viral transformation of cells. No less than six

chapters are either devoted exclusively to this topic or mention it in detail. There are, overall, some 17 chapters on the role of protein phosphorylation in the control of transcription, translation and cell transformation. However, it should not be thought that some of the more established areas have been neglected; about half of the contents deals with protein kinases, phosphoprotein phosphatases and carbohydrate metabolism. There is now considerable knowledge of the structure and catalytic mechanism of cyclic-AMP-dependent protein kinase, which is discussed at length in several articles. The detailed descriptions of this enzyme at the atomic level contrast strongly with the imperfectly understood role of protein phosphorylation in the imperfectly understood eukaryotic nucleus. The books give the clear impression of a subject with distinct, well-established origins now bursting out in many directions.

Reading either whole sections or individual chapters I found much useful information and many stimulating ideas. Almost all of the chapters give an impression of current work being actively pursued, with little that is dated or incorrect. Excellently produced in the clear, uniform style typical of Cold Spring Harbor publications, the books will be equally useful to those new to protein phosphorylation who wish to discover how it relates to their current research interests, and to those already working in the area who need to be informed of new developments. This is undoubtedly a key reference work which should be in any laboratory working on protein phosphorylation, and in all medical and biological libraries. □

P.J. England is a Lecturer in Biochemistry at the University of Bristol.

What can be inferred from exciton lines?

Dennis Dunn

Excitons: Their Properties and Uses. By Donald C. Reynolds and Thomas C. Collins. Pp.291. ISBN 0-12-586580-5. (Academic: 1981.) \$36, £33.80.

THE optical absorption of most insulating crystals exhibits a series of sharp lines at frequencies just below the onset of the continuous absorption spectrum. These are the exciton lines. The non-interacting electron theory presented in most solid state physics texts readily accounts for the general features of the continuous absorption spectrum but fails to predict the existence of the exciton lines. These arise as a consequence of the electron-hole correlation induced by the Coulomb interaction.

The aim of *Excitons* is to provide a self-contained review of the theory and experimental properties of excitons and of the information about electronic structure which can be determined by a study of the exciton lines.

The conventional effective-mass theory is presented in Chapter 2, which includes discussion of the effects of electric and magnetic fields (Stark and Zeeman splitting). The highlights of the book are the chapters on the experimental properties of intrinsic and bound excitons. These, of course, are just the areas where the authors have made significant contributions and their mastery of the subject is clearly demonstrated. Set in between — the chapters seem to be randomly ordered — is a brief review of the recent work on the

electron-hole liquid, a condensed phase which occurs only if the optical excitation is intense enough to produce a high exciton density.

There are a few surprising omissions from the text. There is no discussion of the theory or experimental properties of the line-shapes. The mass of research on excitons in alkali halides rates only one paragraph, and the rare-gas solids are not mentioned. This is a great pity because research on these materials, which have "deep" excitons, has demonstrated that the effective-mass theory has a far wider range of applicability than was previously thought.

The low-point of the book is the first chapter which tries to lay down the theoretical foundations of exciton theory. Here the authors have been too ambitious and have attempted to present the whole of many-body theory in one brief account: Green function formalism, Bethe-Salpeter equations, spectral representations all appear and disappear within the space of ten pages.

Excitons is certain to become one of the standards in solid state literature. Physicists who want to initiate their research students into the mysteries of excitons can safely direct them to this book with just one piece of advice: start with Chapter 2. □

Dennis Dunn is a Lecturer in Theoretical Physics at the University of Reading.

Textbook supplement

Next week's issue of *Nature* will include the Textbook Supplement, comprising reviews of over 100 recently published books for undergraduate students.

BOOKS RECEIVED

Chemistry

- BAILEY, R.T., NORTH, A.M. and PETHRICK, R.A. *Molecular Motion in High Polymers*. Pp.415. ISBN 0-19-851333-X. (Clarendon Press/Oxford University Press: 1981.) £42.50.
- BATES, R.B. and BEAVERS, W.A. *Carbon-13 NMR Spectral Problems*. Organic Chemistry. Pp.259. ISBN 0-89603-010-5. (Humana Press, Clifton, New Jersey: 1981.) Hbk \$29.50; pbk \$12.95.
- BURNS, D.T., TOWNSHEND, A. and CARTER, A.H. *Inorganic Reaction Chemistry. Vol.2, Reactions of the Elements and Their Compounds. Part B: Osmium to Zirconium*. Pp.580. ISBN 0-85312-352-7. (Wiley: 1981.) £27.50, \$61.75.
- CULLIS, C.F. and HIRSCHLER, M.M. *The Combustion of Organic Polymers*. Pp.419. ISBN 0-19-851351-8. (Clarendon Press/Oxford University Press: 1981.) £37.50.
- FIESER, M., DANHEISER, R.L. and ROUSH, W. *Fieser and Fieser's Reagents for Organic Synthesis*. Vol.9. Pp.596. ISBN 0-471-05631-6. (Wiley: 1981.) £28, \$55. Also available as a 9-Vol. set, £225, \$450.
- GOLDWHITE, H. *Introduction to Phosphorous Chemistry*. Pp.113. Hbk ISBN 0-521-22978-2; pbk ISBN 0-521-29757-5. (Cambridge University Press: 1981.) Hbk £12.50; pbk £5.95.
- JEFFERY, P.G. and HUTCHISON, D. *Chemical Methods of Rock Analysis*. 3rd Edn. Pergamon Series in Analytical Chemistry, Vol.4. Pp.379. ISBN 0-08-023806-8. (Pergamon: 1981.) £25, \$60.
- JENNINGS, K.R. and CUNDALL, R.B. (eds). *Progress in Reaction Kinetics*, Vol.10. Pp.406. ISBN 0-08-027155-3. (Pergamon: 1981.) £36, \$82.50.
- MAITLAND, G.C. *et al.* *Intermolecular Forces. Their Origin and Determination*. Pp.616. ISBN 0-19-855611-X. (Clarendon Press/Oxford University Press: 1981.) £39.50.
- MORTIMER, R.G. *Mathematics for Physical Chemistry*. Pp.403. Pbk ISBN 0-02-384000-5. (Collier Macmillan: 1981.) Pbk £6.95.
- SJÖSTRÖM, E. *Wood Chemistry. Fundamentals and Applications*. Pp.223. ISBN 0-12-647480-X. (Academic: 1981.) \$22.
- YINON, J. and ZITRIN, S. *The Analysis of Explosives*. Pergamon Series in Analytical Chemistry, Vol.3. Pp.310. Hbk ISBN 0-08-023846-7; flexi ISBN 0-08-023845-9. (Pergamon: 1981.) Hbk £25; flexi £9.35, \$22.50.

Biological Sciences

- CLARK, M. *Mammal Watching*. Pp.176. ISBN 0-7278-2010-9. (Severn House, London: 1981.) £7.95.
- CORDELL, G.A. *Introduction to Alkaloids. A Biogenetic Approach*. Pp.1055. ISBN 0-471-03478-9. (Wiley: 1981.) £92.50, \$165.
- DAY, C.E. (ed.). *High-Density Lipoproteins*. Pp.728. ISBN 0-8247-1220-X. (Marcel Dekker: 1981.) SwFr. 185.
- DAY, D. *The Doodsbay Book of Animals. A Unique Natural History of Three Hundred Vanished Species*. Pp.288. ISBN 0-85223-183-0. (Ebury Press, London: 1981.) £14.95.
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- DIXON, G.R. *Vegetable Crop Diseases*. Pp.404. ISBN 0-333-23574-6. (Macmillan Press, London & New York: 1981.) £35.
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- DOWNER, R.G.H. (ed.). *Energy Metabolism in Insects*. Pp.244. ISBN 0-306-40697-7. (Plenum: 1981.) \$32.50.
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- PERLMAN, D. (ed.). *Advances in Applied Microbiology*, Vol. 26. Pp.271. ISBN 0-12-002626-0. (Academic: 1980.) \$29.50.
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